then added 1 ml. of sodium bicarbonate solution (1%), 1 ml. of sodium borate solution (satd.), 0.4 g. of boric acid and 2 ml. of potassium iodide solution (2%). The iodine generated was titrated with 0.0200 *N* sodium arsenite (starch indicator). The results of the two determinations are presented in Fig. 3.

Separation of the Intermediates in the Reaction of IIc to IIIc.—In the above experiment the aliquot removed after 40 minutes of reflux was separated by paper electrophoresis in borate buffer, *p*H 6.0, using the apparatus and techniques previously described.[11] The anodic migrations (700 volts, 14 milliamp., 6.0 hours) of the components of the sample are: 40B, −2.5 cm.; 40A, +13.9 cm. These compared with the anodic migrations of 14.0 cm. for IIc and −2.0 cm. for IIIc. A reduced-scale overlay of these results is given in Fig. 4 (1).

Spot 40B was eluted with ethanol from the paper, the solution concentrated and separated into two components, spots M and N, using paper chromatography (isopropyl alcohol–water (7:3), Schleicher and Schuell No. 597 paper, descending technique, 18 hours). The results are shown in Fig. 4 (2).

Spots M and N were eluted from the paper, and the ultraviolet spectra of their solutions determined. The results are shown in Fig. 5.

Acknowledgments.—The authors wish to express their appreciation to Dr. Aaron Bendich for many useful discussions. They are grateful to Dr. George B. Brown for helpful suggestions and continued encouragement.

[CONTRIBUTION FROM THE LABORATORY OF THE CHILDREN'S CANCER RESEARCH FOUNDATION AND HARVARD MEDICAL SCHOOL, BOSTON 15, MASS.]

The Deuterium Exchange of Water-soluble Polypeptides and Proteins as Measured by Infrared Spectroscopy[1,2]

BY E. R. BLOUT, C. DE LOZÉ AND A. ASADOURIAN

RECEIVED DECEMBER 19, 1960

An infrared spectroscopic method is described which measures the rate of hydrogen → deuterium exchange of the secondary amide hydrogens of polypeptides and proteins. This method involves solution of the material in D_2O and the measurement of the time-dependent decrease in the intensity of the amide II absorption band around 1550 cm.$^{-1}$. To calibrate the measurements the deuterium exchange of poly-α,L-glutamic acid in helical and random conformations has been studied. It was found that the random coil conformation exchanges very rapidly (within ten minutes) whereas the helical conformation requires many hours for the complete exchange of the amide hydrogens for deuterium. Application has been made of this method to glutamic acid:lysine copolymers and to ten globular proteins from which estimates of the percentage hard-to-exchange amide hydrogens have been obtained. The hard-to-exchange amide hydrogens in these proteins range from less than 10% to about 60% of the backbone peptide hydrogens; thus there are proteins in which there are more hard-to-exchange amide hydrogens than the percentage helix, estimated from optical rotatory dispersion. Some suggestions are made as to the cause of the differences in the results obtained with the two methods.

Introduction

It has been known for some time that hydrogen atoms attached to nitrogen and oxygen in proteins are replaced by deuterium when such materials are dissolved in D_2O solutions. Indeed, several years ago we observed[3] that some of the amide hydrogens in bovine serum albumin and in ovalbumin exchanged rapidly with D_2O, whereas others required heating or alkaline treatment to effect exchange. On the basis of these observations the existence of two different types of amide groups in these proteins was postulated. We have now examined the rate of deuterium exchange for a series of water-soluble polypeptides of known composition and conformation and correlated these results with the conformation of these macromolecules as determined by optical rotation. In addition, we report new results on the rates and extent of deuterium exchange of a series of globular proteins. From these experiments we have determined the percentage "hard-to-exchange amide hydrogens" (HEAH) in these proteins.

In his work on the determination of the secondary structure of proteins, Linderstrøm-Lang and his co-workers[4] have examined the exchange reaction of insulin, ribonuclease and myoglobin with D_2O, using cryosublimation followed by density determination of the water removed to measure the amount of hydrogen → deuterium exchange. Some additional experiments on ribonuclease using similar techniques have recently been reported.[5] Haggis[6] has determined the exchange of deuterium for amino *and* amide hydrogen in certain proteins and nucleoproteins in the solid state using the infrared absorption band due to the NH stretch of such groups, which lies at 3300 cm.$^{-1}$. In addition, preliminary notes on the deuterium exchange of some polypeptides in non-aqueous systems have appeared.[7] Finally, mention should be made of the work of Fraser and MacRae[8] who studied hydrogen → deuterium exchange reactions in fibrous proteins in the hydrogen overtone region in the near infrared.

In our laboratory we have been particularly concerned with the determination of the structure of high molecular weight polypeptides in aqueous

(1) This is Polypeptides XXXIV. For the preceding paper in this series see L. Stryer and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 1411 (1961). Alternate address for E. R. Blout, Chemical Research Laboratory, Polaroid Corp., Cambridge 39, Mass.

(2) This work was supported in part by U. S. Public Health Service Grant A2558 and by the Office of the Surgeon General, Department of the Army.

(3) H. Lenormant and E. R. Blout, *Nature*, **172**, 770 (1953); *Bull. Soc. Chim. Fr.*, 859 (1954).

(4) (a) For previous references see K. U. Linderstrøm-Lang, Chapter in "Symposium on Protein Structure," edited by A. Neuberger, Methuen and Co., Ltd., London, 1958, p. 23; (b) E. S. Benson, *Compt. rend. Lab. Carlsberg, ser. chim.*, **31** (16), 235 (1959).

(5) C. L. Schildkraut and H. A. Scheraga, *J. Am. Chem. Soc.*, **82**, 58 (1960).

(6) G. H. Haggis, *Biochim. Biophys. Acta*, **23**, 494 (1957).

(7) (a) A. Elliott and W. E. Hanby, *Nature*, **182**, 654 (1958); (b) D. G. H. Ballard, C. H. Bamford and A. Elliott, *Macromol. Chem.*, **35**, 222 (1960).

(8) (a) R. D. B. Fraser and T. P. MacRae, *J. Chem. Phys.*, **28**, 1120 (1958); (b) **29**, 1024 (1958).

environments, inasmuch as this is the natural milieu for proteins. Infrared techniques appear to be suited for such determinations, since it is possible in the infrared region to differentiate the absorption of the amide NH groups from amino groups such as the ϵ-amino groups of lysine, as well as from hydroxyl groups such as those in tyrosine, serine and threonine. In the method described in this paper we examine the rate of disappearance of the amide II band which lies in the 1525 to 1560 cm.$^{-1}$ region and is characteristic of the secondary amide (peptide) groups. This is a complex band which has its origin in a coupled CN vibration and NH deformation frequency.[9]

We have chosen to observe changes in the amide II (1550 cm.$^{-1}$) band upon deuteration rather than the NH stretching frequency at 3300 cm.$^{-1}$. If the 3300 cm.$^{-1}$ band were used it would be very hard to perform experiments in aqueous solution since it is difficult to differentiate the amide NH stretching mode from other types of hydrogenic stretching modes. Furthermore, the amide II band lies in the region where D_2O is relatively transparent, and where HOD shows little absorption.[10] Therefore we are able to measure the spectra (in this region) of polypeptides and proteins at reasonable concentrations in D_2O solution.[11]

Upon deuteration of the polypeptide or protein, the amide II band shifts from about 1550 to 1450 cm.$^{-1}$.[12] Theoretically it should be possible to use the increase in absorption at 1450 cm.$^{-1}$ as a measure of the extent of deuteration, and we have made such measurements in some cases. However, the overlap by the CH deformation bands in this region make this measurement less suitable than the determination of the disappearance of the amide II (1550 cm.$^{-1}$) band.

Experimental

Materials.—The sample of poly-α,L-glutamic acid, the various copolymers of L-glutamic acid and L-lysine were prepared by methods previously described.[13,15] The polyglutamic acid sample, #S2035–186B, had an intrinsic viscosity of 1.88 in 0.2 M sodium chloride solution at pH 7.3, from which an MW_w of 80,000 is estimated. For the glutamic acid : lysine copolymers the sample numbers, mole ratio, intrinsic viscosity in 0.2 M salt at pH 3 and estimated weight average molecular weights (MW_w) are given in Table I.

The proteins were commercial preparations whose sources and lot numbers are (WBC, Worthington Biochemical Corp.; AL, Armour Laboratory; NBC, Nutritional Biochemical Corp.; BC, Borden Co.; S, Squibb; ELC, Eli Lilly Co.): ribonuclease WBC, lot 565; chymotrypsin WBC, lot CD-576-81; lysozyme WBC, lot 570; trypsin WBC, lot 62–330; chymotrypsinogen WBC, lot 660–61; ovalbumin Al, lot E90115, and NBC; bovine plasma albumin AL, lot 29–633; β-lactoglobulin BC, preparation 40/17 and NBC; γ-globulin AL, lot 1823; insulin (Zn salt) S and ELC, lot T23344. We would like to thank Dr. Otto K. Behrens of Eli Lilly and Co., Indianapolis, Ind., for the gift of this sample.

TABLE I

COPOLYMERS OF L-GLUTAMIC ACID:L-LYSINE

Sample	Mole ratio L-Glu:L-Lys	$[\eta]$	MW_w
MI-407-200B	7:3	1.5	110,000
MI-432-200	6:4	0.53	36,000
MI-430-200	5:5	.74	42,000
MI-500-200B	4:6	.72	39,000

Method. Preparation of Solutions.—The polypeptide samples were dried prior to solution for 2 hr. at 100° *in vacuo*. Proteins were dried for 2 hr. at lower temperature (50°) in order to minimize changes in structure. The enzymatic activity of trypsin was tested after such treatment and found to be unchanged as compared with undried samples. The concentrations of all the solutions in D_2O were about 5%. The determinations of deuterium exchange in polyglutamic acid were made in a 1:1 D_2O–dioxane system since this material is insoluble in D_2O at low pH's. All the proteins, the copolymers and the sodium salt of polyglutamic acid were dissolved in D_2O; when necessary the pD was adjusted by the use of DCl. The starting time of each experiment was marked from the instant when the solvent was added to the dry sample. In many cases solution was not immediate and thus an accurate estimate of the rate of deuteration during the first 10 minutes was often impossible. The D_2O solutions were usually inserted into the cells after 2 to 3 minutes, even if solution was not complete; the portion of the sample which was not dissolved was not used in the experiment. Therefore the concentrations of polypeptides and proteins measured was sometimes somewhat less than 5%. In the experiments reported here the error due to the finite time for solution was considered negligible after 10 minutes.

Spectral Determinations.—The infrared spectra were determined with a Perkin–Elmer double-beam spectrometer (model 21) using a sodium chloride prism and calcium fluoride cells having a thickness of 0.05 mm. Most of the spectra were run at speeds of about one second per cm.$^{-1}$ over the region 1400–1800 cm.$^{-1}$. This speed allows only one determination every 8 minutes, but when faster scanning speeds were tried errors in the estimation of the band intensities were introduced. The estimated resolution was about 5 cm.$^{-1}$ at 1600 cm.$^{-1}$.

Preparation of the Calibration Curve.—In order to determine that there is a linear relationship between the amide I and amide II band intensities in polypeptides we have measured the ratio of the optical density at the absorption maxima of these bands at various concentrations in a model compound. As a model substance for this purpose we have chosen the synthetic polypeptide poly-α,L-glutamic acid which exists at low pH in a helical conformation. Solutions of polyglutamic acid ranging in concentration from 0.625 to 5% in 1:1 D_2O–dioxane at pD 4 were measured. Since, as will be seen below, this polypeptide exchanges its amide hydrogens for deuterium very slowly, this is a good compound for the preparation of such a calibration curve. In addition, the spectra of the above solutions were run as soon as possible after solution occurred in order to minimize any deuterium–hydrogen exchange. The optical density of the amide I band was then plotted against that of the amide II band and the data are shown in Fig. 1. The slope of the line is approximately 0.4 and the relationship is obviously linear over the concentration range measured.

From the calibration curve it is possible to determine the amide II optical density (b) for an undeuterated polypeptide at any concentration knowing only the optical density of the amide I band (a). Furthermore, if we assume that the ratio of the optical density of the amide II band to the amide I band is the same in proteins as in polypeptides (which is justified, as will be seen below) then this calibration curve can be used for proteins also.

Estimation of Band Intensities.—The spectra were recorded on logarithmic paper and thus the optical densities could be read directly when the base line of the spectrum coincided with the zero line (see for example Fig. 2). When

(9) (a) R. D. B. Fraser and W. Price, *Nature*, **117**, 419 (1952); (b) T. Miyazawa, T. Shimanouchi and S. Mizushima, *J. Chem Phys.*, **29**, 611 (1958); (c) T. Miyazawa and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 712 (1961).

(10) E. R. Blout and H. Lenormant, *J. Opt. Soc. Am.*, **43**, 1093 (1953).

(11) Since this paper was prepared, Nielsen, *et al.*, have published two preliminary notes describing deuterium exchange measurements of small peptides and poly-D,L-alanine using a similar technique; S. O. Nielsen, W. P. Bryan and K. Mikkelsen, *Biochim. Biophys. Acta*, **42**, 550 (1960), and W. P. Bryan and S. O. Nielsen, *ibid.*, **42**, 552 (1960).

(12) H. Lenormant, *Ann. Chim.*, [12] **5**, 459 (1950).

(13) E. R. Blout and M. Idelson, *J. Am. Chem. Soc.*, **78**, 497 (1956); P. Doty, A. Wada, J. T. Yang and E. R. Blout, *J. Poly. Sci.*, **23**, 851 (1957); M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **80**, 4631 (1958).

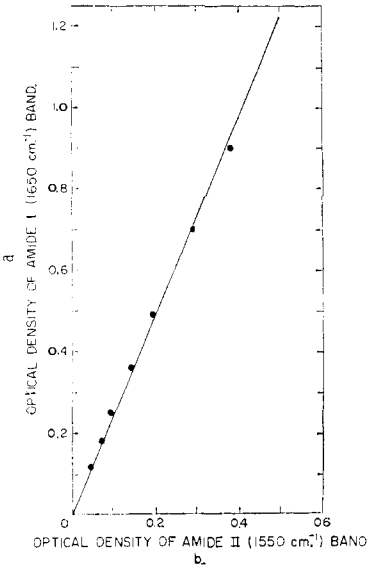

Fig. 1.—The relationship between the optical densities of amide I and amide II bands of poly-α,L-glutamic acid at various concentrations in dioxane–D_2O solution (1:1).

this was not the case the base line of the amide I band was drawn as a line parallel to the zero line from the optical density at 1800 cm.$^{-1}$. The base line of the amide II band is sometimes more difficult to determine. In certain cases (see Fig. 5, chymotrypsinogen) the base line of the amide II band obviously does not coincide with the one chosen for the amide I band probably because the scatter due to the solution is not the same at 1550 cm.$^{-1}$ as at 1800 cm.$^{-1}$. Another difficulty in the determination of the amide II base line arises from the fact that the proteins contain carboxyl groups which were in part ionized at the *p*D where the spectral determinations were made. Thus the infrared absorption band due to the COO^- antisymmetric stretch around 1575 cm.$^{-1}$ overlaps the amide II band at 1550 cm.$^{-1}$ *e.g.*, Fig. 6, lysozyme. In such cases the base line of the amide II band was determined from the spectra of the protein after complete deuterium exchange had occurred; see Fig. 2. It is recognized that the accuracy of this procedure is low and probably provides the major source of error in the determinations. It should also be pointed out that the amide II band becomes quite weak during the last stages of deuterium exchange and thus the estimation of its intensity at those times is difficult and involves some error.

Calculation of Percentage HEAH at Ten Minutes.—We have arbitrarily chosen 10 minutes as the time for the maximum of the percentage hard-to-exchange amide hydrogens (% HEAH) in the polypeptide and protein solutions because the deuterium exchange of a completely random polypeptide is complete within this time interval and also very little (if any) deuteration has occurred in a completely helical polypeptide having "hard-to-exchange amide hydrogens" during this same period (see Fig. 3). If at 10 minutes *a* is the optical density of the amide I band of a polypeptide or protein solution, it is possible to obtain from the calibration curve (Fig. 1) the related optical density (*b*) of the amide II band for a completely helical polypeptide. The actual value (*b'*) of the amide II band observed for polypeptides and proteins is always smaller than *b* (see Figs. 1, 5, 6). In order to determine the percentage HEAH of the sample compared with a helical polypeptide, use is made of the equation

$$\% \text{ HEAH} = (b'/b) \times 100$$

This same calculation can be applied at any time during the deuterium exchange reaction. However, as has already been pointed out, the values obtained toward the end of the exchange are more subject to error because of the low absorption due to the amide II band. When the % HEAH after 24 hr. was less than 5% and when any very weak band (optical density less than 0.015) did not disappear upon heating, the exchange was considered complete.

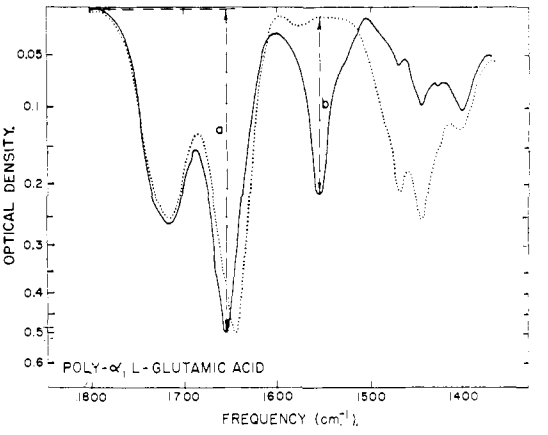

Fig. 2.—The infrared spectra of poly-α,L-glutamic acid, ———, and completely deuterium-exchanged poly-α,L-glutamic acid, ...; both spectra determined at 5% concentration in 1:1 D_2O–dioxane solution.

Rates of Deuteration.—Curves of the rates of deuteration have been obtained by plotting the ratio of the amide II optical density to that of the amide I as a function of time. We have chosen to plot this ratio because it is comparable for any sample and independent of concentration. The temperature of the room in which the spectral determinations were made was 24 ± 1°. However, heating of the cell during spectral determinations may have raised the temperature of the solutions to as high as 32°.

Results and Discussion

Polyglutamic Acid.—An ideal model compound for use in deuterium exchange studies would be a synthetic polypeptide having the α-helical conformation in water. Since at the present time we know of no completely helical synthetic polypeptide which dissolves in water alone at high concentrations, we have chosen as a model compound for this study poly-α,L-glutamic acid (PGA). This polypeptide is soluble in water–dioxane mixtures and much work on its conformation has been performed using infrared spectroscopy and optical rotation.[13] Through these methods it has been shown that PGA in its un-ionized state exists in a helical conformation both as a solid and in dioxane–water solutions, whereas the sodium salt of poly-α-L-glutamic acid (SPG) exists in a random conformation which may be oriented to give an extended β-conformation in the solid state.

The infrared spectrum of the helical (un-ionized) form of poly-α,L-glutamic acid is shown in Fig. 2 along with the spectrum of this same form after complete exchange of its amide hydrogens with deuterium. It should be noted that the amide I band does not change in intensity upon deuteration but shifts to lower frequency by about ten wavenumbers. Upon deuteration the amide II band at 1550 cm.$^{-1}$ is lost completely and deuterated amide II bands appear around 1450 cm.$^{-1}$.

The rates of disappearance of the amide II bands for PGA, SPG and two intermediate ionization states of this polypeptide have been plotted as a function of time in Fig. 3. It is quite clear from these data that the rate of exchange of the amide hydrogens is very much slower for helical PGA than

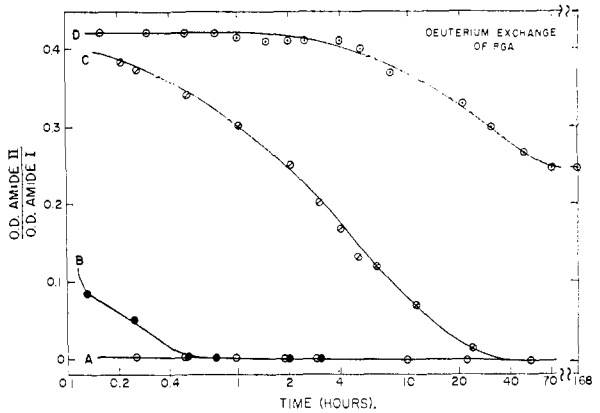

Fig. 3.—Rates of deuteration of poly-α,L-glutamic acid: curve A, O-O-O, *p*D 7 (5% in D_2O); curve B, ●-●-●, *p*D 5.7 (5% in D_2O–dioxane 1:1); curve C, ⊖-⊖-⊖, *p*D 4.2 (5% in D_2O–dioxane 1:1); curve D, ⊙-⊙-⊙, *p*D 3.5 (5% in D_2O–dioxane 1:1).

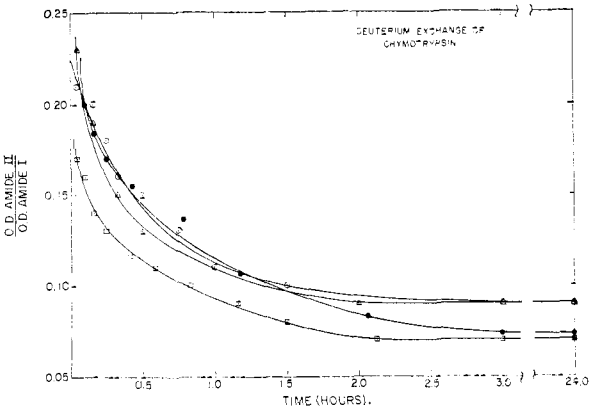

Fig. 4.—Rates of deuteration of chymotrypsin ~5% in D_2O solution. The four different curves represent data from four different experiments.

for random coil SPG.[14] With SPG the hydrogen–deuterium exchange is complete in less than ten minutes at room temperature (Fig. 3, curve A). With PGA at *p*D 4.2, in which optical rotatory dispersion data indicate a helical content of 100%,[13] the complete deuterium exchange requires at least 24 hours (Fig. 3, curve C).

A special case seems to arise with PGA at *p*D 3.5, whose infrared spectrum exhibits a very strong band around 1705 $cm.^{-1}$. This band is probably due to a carboxyl–carboxyl interaction of the side chains. If the *p*D 3.5 solution is heated for 6 minutes at 50° no deuterium exchange occurs, but the spectrum becomes similar to that shown in Fig. 2. Deuterium exchange determinations on a sample heated in this manner show that exchange is very slow and is not complete after 168 hours (Fig. 3, curve D). However, complete deuterium exchange can be effected at this time by heating at 60° for 10 minutes.

Copolymers of L-Glutamic Acid and L-Lysine.—Having established that the helical conformation of a synthetic polypeptide shows a very much lower rate of exchange of its amide hydrogens with deuterium than does a random coil conformation, we next examined a series of high molecular weight, water-soluble copolymers of L-glutamic acid and L-lysine.[15] Optical rotatory dispersion measurements of these materials indicated that they existed partially in a helical conformation.[15] The results of the deuterium exchange experiments are shown in Table II in which an estimate of the percentage "hard-to-exchange amide hydrogens" (HEAH) is given along with the estimate of the percentage helix for each compound as determined by optical rotatory dispersion. The determinations by the two methods are comparable except for the 7:3 copolymer.

(14) It should be noted that the presence of dioxane in these solutions certainly affects the rate of deuterium exchange—probably decreasing it to a certain extent—especially for the helical conformation. Thus the deuteration curve of PGA in dioxane–D_2O cannot be compared directly to deuteration curves of materials dissolved in D_2O alone.

(15) E. R. Blout and M. Idelson, *J. Am. Chem. Soc.*, **80**, 4909 (1958).

Proteins.—The infrared–deuterium exchange technique has been applied to several globular proteins in order to determine the percentage HEAH in these materials. In Fig. 4 there is plotted the ratio of the optical density of the amide II band to the optical density of the amide I band as a function of time for D_2O solutions of chymotrypsin. The four curves are the data from four different experiments, the variation among the data indicating the reproducibility. It is clear that the deuterium exchange reaction has reached equilibrium after about three hours. The shape of these curves and those for other proteins are similar; the time for deuteration equilibrium is also similar.

TABLE II

Mole ratio	Approx. *p*D[a]	% HEAH (10 min.)	Time required for complete H → D exchange	Helix, % detd. by opt. rot. at approx. same *p*H
	Poly-α,L-glutamic acid			
	3.4[b]	100	46 hr.	100
	4.2[b]	100	24 hr.	100
	5[b]	100	Not detd.	>90
	5.7[b]	20	2 hr.	44
	7	0	<10 min.	0
	Copolymers of L-glutamic acid:L-lysine			
7:3	3	65–70	1 hr.	>90
6:4	3	65–70	45 min.	70
5:5	3	45	45 min.	50
4:6	3	25	45 min.	25

[a] The *p*D's were measured using narrow range indicator paper and therefore are only approximate. The optical rotation data were determined in water or water–dioxane solutions at approximately the same *p*H. The differences between *p*H's and *p*D's can be significant in the sharp region of the titration curve of PGA, and could explain for instance the discrepancies observed in the data for PGA at *p*D 5.7. [b] Solutions so marked in 1:1 dioxane:D_2O; all other solutions in D_2O.

From data such as those in Fig. 4 calculations have been made of the percentage HEAH for the various proteins. Before discussing these estimates it is necessary to mention several problems associated with the measurements. First, as pointed out in the Experimental section, it is necessary to determine the optical density of the amide

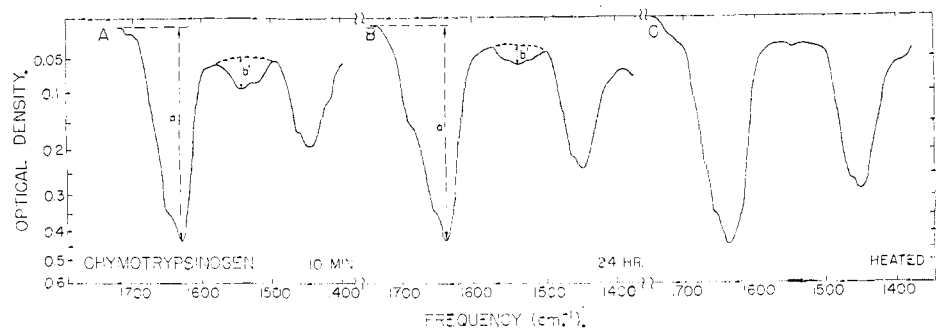

Fig. 5.—Infrared spectra of chymotrypsinogen in D_2O solution: curve A, at 10 min.; curve B, at 24 hr.; curve C, the same sample as in curve B heated 20 min. at 50°.

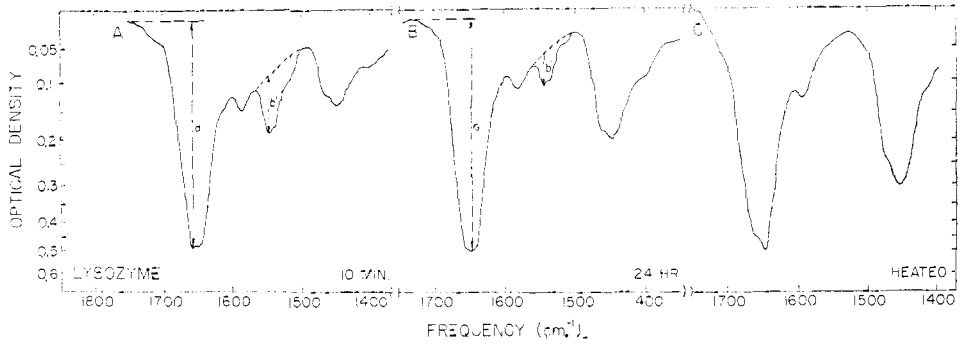

Fig. 6.—Infrared spectra of lysozyme in D_2O solution: curve A, at 10 min.; curve B, at 24 hr.; curve C, the same sample as in curve B heated 2 hr. at 65°.

II band before deuteration and after deuteration as accurately as possible. This is often difficult because the absorption curve does not approach zero optical density even upon complete deuteration. This may be caused by the presence of overlapping bands in this region. Chymotrypsinogen (Fig. 5) is an example of a protein which shows little or no ionized carboxyl (1575 cm.$^{-1}$) in its spectrum. On the other hand, lysozyme (Fig. 6) run at pD ~5 does show ionized carboxyl which makes the estimate of the zero line of the deuterated material more difficult. Secondly, in each case, at the end of 24 hr., there is a residual absorption due to amide groups which have not yet exchanged. For an accurate determination of the zero line it was thus necessary to force the exchange by heating the solution at the end of 24 hr. The temperature of heating varied from 35 to 65° according to the protein and was chosen so that no precipitation or gel formation occurred. The time of heating varied from 10 to 60 minutes. The heating resulted in the complete disappearance of the amide II band at 1550 cm.$^{-1}$ as can be seen from Figs. 5 and 6, and the spectrum of the heated solution was used for the determination of the base line for the estimation of the change in optical density of the amide II band.

Finally it is necessary to know that the optical density of the amide II bands in proteins bears the same relationship to their amide I bands as does the amide II bands in our model polypeptides. This could be done by preparing a calibration curve for each protein using films of non-deuterated proteins of different thicknesses and plotting the optical densities of the amide I band *vs.* that of the amide II band in a manner similar to that for polyglutamic acid solutions. This has been done for several proteins and we find the slope is close to that obtained with the PGA calibration curve.[16] Thus the initial optical density of the amide II bands of proteins can be assumed to be about the same as that for polyglutamic acid at the same concentration and we are justified in the use of PGA calibration curve (Fig. 1) for protein solutions.

As can be seen from Fig. 4 and the calculated initial optical density ratio (0.4 to 0.5), the measurement of the optical density of the amide II band at 10 min. is performed at a time after the initial rapid exchange of some amide hydrogens for deuterium. This rapid H → D exchange is presumably due to the deuteration of the random regions of the polypeptide chains. The region of slower exchange of deuterium for amide hydrogen which occurs in the time interval 10 minutes to 3 hours is presumably due to the deuteration of the helical portions of the polypeptide chain. At the end of 24 hours many of the proteins show that a percentage of their amide hydrogens have not been exchanged for deuterium. At this time we are not able to assign this core to a particular structure, but it is noteworthy that heating the solution in all cases induces complete H → D exchange. Perhaps these portions of the molecules resistant to H → D exchange are highly hydrophobic regions.

(16) It should be pointed out that a determination of the band area would be more accurate than a simple estimation of their optical densities. This is especially true in the cases of proteins, where the amide II band is broad, probably because several structures are present (helical, random, etc.). However such an area estimation does not seem to be required because of the implicit experimental errors in these measurements of deuterium exchange.

As may be seen from Table III, the calculations of the percentage HEAH for some ten proteins shows a variation in the percentage HEAH from less than 10% for γ-globulin to 60% for insulin. It can be concluded from these data that, on the one hand, γ-globulin contains a very small amount of strongly-hydrogen-bonded amide groups whereas insulin has more than one-half of its amide groups strongly hydrogen-bonded or in hydrophobic regions. As indicated in the table the average deviation for a series of measurements is slightly less than ±5%.

TABLE III

Protein	Approx. *p*D[a]	% HEAH[b] (10 min.)	Av. dev., %	Number of detn.	% HEAH (24 hr.)
Insulin	2	60	±2	3	12
Ovalbumin	5	50	±4	4	20
Lysozyme	4.5–5	45	±5	4	15
Chymotrypsin	4.5	43	±7	4	15
Bovine plasma albumin	5	40	±4	3	<5
Ribonuclease	4.5	35	±6	4	12
β-Lactoglobulin	5–5.5	25	±5	3	10
Chymotrypsinogen	4	20	±6	3	10
Trypsin	4	14	±4	3	5
γ-Globulin	4	<10		4	<10

[a] The *p*D's were measured using narrow range indicator paper. It is possible that the rate of deuterium exchange in proteins is highly *p*D dependent, even where no change of conformation occurs. We have tried to make all the determinations between *p*D 4 and 5, except in the case of insulin which is not soluble at this *p*D. [b] From the data given by Linderstrøm-Lang, *et al.*,[4] we have calculated the percentage HEAH they found at the beginning of deuterium exchange (using another method) to have been: insulin, 66; ribonuclease, 45.

If we compare the data in Table III with estimates of the excess right-handed helical contents obtained for the same proteins by optical rotatory dispersion,[17] we note that in general the infrared–deuterium exchange method shows higher values. A somewhat better agreement is shown between the deuterium exchange data and the estimates obtained from rotation measurements at the sodium D line.[18] At this time we offer little in the way of definitive explanations for the differences observed between the results obtained with optical rotation and those with deuterium exchange. With the infrared technique described herein we do not make the tacit assumption that the "hard-to-exchange amide hydrogens" necessarily represent the helical conformation of polypeptides and proteins, although we use a helical polypeptide as a model for a substance containing "hard-to-exchange amide hydrogens." The low values obtained with rotatory dispersion measurements may be caused by the fact that this method measures only the excess of one sense of helix over the other sense of helix and polypeptide chains with both sense of helix may exist in proteins. Alternative explanations of these data are that in proteins in addition to the hard-to-exchange amide hydrogens associated with helical portions of the polypeptide chains there are other regions of hard-to-exchange amide hydrogens. These may lie in β-structures or in areas of the polypeptide chain so surrounded by hydrophobic bonds that the deuterium exchange reaction is very slow.

Acknowledgment.—We wish to thank Dr. D. B. Wetlaufer and D. T. Miyazawa for many interesting discussions.

(17) P. Doty, *Rev. Mod. Phys.*, **31**, no. 1, 107 (1959).

(18) C. Schellman and J. A. Schellman, *Compt. rend. Lab. Carlsberg, ser. chim.*, **30** (26), 463 (1958).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL SCIENCES, STANFORD RESEARCH INSTITUTE, MENLO PARK, CALIFORNIA]

Potential Anticancer Agents.[1] LIV. Synthesis of 3-Amino-2,3-dideoxy-β-D-ribofuranosides *via* the 2,3-Episulfonium Ion Approach

BY CHARLES D. ANDERSON,[2] WILLIAM W. LEE, LEON GOODMAN AND B. R. BAKER

RECEIVED NOVEMBER 10, 1960

Application of a synthetic sequence involving migration of an ethylthio group *via* an episulfonium intermediate led to a synthesis of 3-amino-2,3-dideoxy-D-ribose isolated as its hydrochloride XVII. The 3-ethylthio glycoside I was converted to its 5-O-trityl derivative V, then treated with methanesulfonyl chloride to give the chloroglycoside VIII. Azidolysis of the chloro compound VIII gave the mixture of azides IX which was reduced to the amine mixture XIII. Acetylation of XIII and fractional crystallization of the mixture gave both acetamidoethylthiofuranosides XI and XVI with the former, originating from opening of the episulfonium ion intermediate at C.3, as the very predominant isomer. Desulfurization of the two isomers XI and XVI gave the crystalline acetamido dideoxy isomers X and XV, whose assumed structures were shown to be correct by analysis of their proton magnetic resonance spectra. A two-step hydrolysis of X gave first the free sugar, 3-acetamido-2,3-dideoxy-D-ribose (XIV) which, in turn, was converted to the amino dideoxy sugar (XVII).

Two previous papers in this series described the synthesis of 2-deoxy-β-D-ribofuranosides[3] and of 2′-deoxyadenosine[4] both utilizing 2,3-episulfonium ions as key intermediates. These successful syntheses demonstrated the utility of this episulfonium approach for the preparation of unnatural

(1) This work was carried out under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Public Health Service, Contract No. SA-43-ph-1892. The opinions expressed in this paper are those of the authors and are not necessarily those of the Cancer Chemotherapy National Service Center. For the preceding paper in this series, *cf.* J. I. DeGraw, L. O. Ross, L. Goodman and B. R. Baker, *J. Org. Chem.*, **26**, in press (1961).

(2) Pacific Lutheran College, Tacoma 44, Washington.

(3) C. D. Anderson, L. Goodman and B. R. Baker, *J. Am. Chem. Soc.*, **81**, 898 (1959).

(4) C. D. Anderson, L. Goodman and B. R. Baker, *ibid.*, **81**, 3967 (1959).