Hydrogen–Deuterium Exchange of Bovine Plasma Albumin*

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ABSTRACT: An improved method for studying hydrogen–deuterium exchange of proteins dissolved in D_2O has been developed. Certain errors inherent in the original Linderstrøm-Lang method have been minimized and some of the experimental techniques made simpler by use of quantitative infrared analysis for the determination of small amounts of D_2O in H_2O. The method has been applied to a study of the N–F isomerization of bovine plasma albumin. Exchange curves have been obtained in the pD region 2.6–5.9. Analysis of the results leads to a picture of the N–F isomerization in which both N and F forms have two regions of slowly exchanging hydrogens. When isomerization of an N form occurs, the more rapidly exchanging region grows at the expense of the more slowly exchanging region. From the pD dependence of the exchange kinetics it can be concluded that the rates of exchange in the isomerization region are determined by the rates of transconformational openings of protein structure. These results support the microheterogeneity model of Foster *et al.* (1965) (Foster, J. F., Sogami, M., Peterson, H. A., and Leonard, W. J. (1965), *J. Biol. Chem. 240*, 2495).

The importance of hydrogen exchange as a method for the study of protein conformation was first appreciated by Linderstrøm-Lang and coworkers (Hvidt *et al.*, 1954; Krause and Linderstrøm-Lang, 1955; Hvidt and Linderstrøm-Lang, 1955). Since then a number of studies of hydrogen exchange of various proteins have appeared (Hvidt and Nielsen, 1966). In his original methods for study of such exchange Linderstrøm-Lang used density gradient tubes for analysis of the amount of D_2O in H_2O isolated from exchanging solutions. The rate of exchange can be followed by changes in infrared transmission when a protein is dissolved in D_2O (Blout *et al.*, 1961; Hvidt, 1963). Nuclear magnetic resonance has also been used to follow exchange in D_2O (Wishnia and Saunders, 1962). Tritium can be used as a tracer in studies of exchange. Modification of the original Linderstrøm-Lang techniques for water isolation (Leach and Springell, 1962; Leach and Hill, 1963) or gel filtration (Englander, 1963) can be used.

Two errors which were not fully appreciated at the time of the development of the Linderstrøm-Lang method (Krause and Linderstrøm-Lang, 1955) are inherent in the method. The first of these is the possibility for further exchange between ice and the exchanging substance during the lyophilization required for isolation of the water for analysis. Thus the method gives erroneous results for simple peptides (Nielsen *et al.*, 1960) poly-DL-alanine (Bryan and Nielsen, 1960) early exchange in ribonuclease (Leach and Springell, 1962) and early exchange in bovine plasma albumin (Hallaway and Benson, 1965). The second error is the possibility that exchange between dry protein and water vapor can occur during the lyophilization step when conditions are such that a fairly high pressure of water vapor is present. Both of these errors can give rise to values for extent of exchange which are systematically too high. Such errors appear to be most serious for those classes of hydrogens which exchange fairly rapidly.

Another difficulty of the Linderstrøm-Lang method is the necessity of heating the fully deuterated protein to approximately 60° in order to remove the last traces of D_2O from the protein prior to addition to H_2O and subsequent exchange. The danger of heat denaturation is thus introduced.

Although the density gradient technique can give a very accurate analysis of D_2O in H_2O and can be used with very small water samples, the difficulties of the method have perhaps discouraged more general use of the technique in studies of protein structure.

In this paper we report a modified method for measurement of exchange which minimizes exchange with ice, does not necessitate heating and utilizes an infrared method for D_2O analysis. The method gives results of precision comparable to the Linderstrøm-Lang method and is a good deal simpler in operation. A disadvantage is that a larger water sample, and therefore more protein, is required.

We have chosen to study the exchange behavior of bovine plasma albumin in acid solution with particular reference to the much studied N–F transition (Foster, 1960; Foster *et al.*, 1965). Fragmentary work on hydrogen exchange of bovine plasma albumin has been presented by Bresler (1958) and Blout *et al.* (1961). More comprehensive work has been reported by Benson *et al.* (1964). These workers, using conventional Linderstrøm-Lang techniques, studied exchange in H_2O at a number of pH values. Two days of exchange at 25° and early exchange at 0° were reported. A limited study of exchange in the N–F transition region was reported by Foster *et al.* (1965). These results supported Foster's microheterogeneity model for the protein. In our work we have made a more detailed study of hydrogen–deuterium exchange of bovine plasma albumin in D_2O at 30° and at pD values between 5.9 and 2.6. A preliminary report of our methods and results has already been presented (Hvidt and Nielsen, 1966).

Experimental Section

All experiments reported here were performed using the same sample of crystalline bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, England, Lot EN 1374).

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A large sample was dissolved in deionized water and lyophilized. Samples of this preparation were used in all exchange runs. No attempt was made to remove fatty acid impurities which were undoubtedly present in the preparation. Chen (1967) has recently discussed fatty acid impurities in plasma albumin and their removal.

An approximately 50-mg sample of lyophilized bovine plasma albumin was added to a weighed test tube and dried to constant weight in a high vacuum system; 1–2-hr drying time was sufficient. After drying air was admitted to the high vacuum chamber and the tube was quickly capped and weighed. To start the exchange 2.500 ml of a 0.15 M solution of NaCl in 99.7% D_2O, containing varying small amounts of concentrated HCl, was added to the protein. The lyophilized protein quickly dissolved. All exchange experiments were carried out at 30.0° Samples of 0.500 ml were removed from the exchanging solution at various times from approximately 15 min to approximately 24 hr after the start of the reaction. Each sample was added to a 3-ml pear-shaped flask with a standard taper male joint mouth. The flask was then sealed with a standard taper cap using Apiezon L grease. The solution in each flask was shell frozen by rotating the flask in a Dry-Ice–alcohol bath. After freezing the samples were stored in a Dry-Ice-filled dewar flask until removal of the D_2O. Generally four samples were taken during each exchange run and the remainder of the exchanging solution reserved for a pD determination. Values of pD_{app} were determined using the method of Mikkelsen and Nielsen (1960). The value of pD is given by

$$pD = pD_{app} + 0.44 \quad (1)$$

where pD_{app} is the apparent value of pD obtained with ordinary glass and calomel electrodes.

When the samples from a run had been collected, they were treated by the following procedure. Each frozen sample was taken and the mouth of the sample flask was immersed in warm water for a few seconds. This enabled removal of the cap and quick wiping away of the remaining Apiezon grease. After the cap had been removed from a flask, the flask was quickly recapped with a clean dry cap and placed back in the Dry Ice to refreeze any of the exchanging mixture which might have melted during the manipulation. After all the sample flasks had been treated in this way the dry caps were quickly removed, the sample flasks placed in a special holder, and the holder was placed in the high vacuum chamber. The chamber was immediately evacuated and the D_2O was removed by continuous mechanical pumping through a Dry-Ice–alcohol trap. A small amount of H_2O formed by condensation on the outside of the sample flasks was always introduced along with the sample flasks, but this amount of H_2O appears to be negligible in comparison with the amount of D_2O present in the samples themselves.

The vacuum chamber consisted of a 2-l. bell jar sealed by means of an O ring to a perforated brass plate which was in turn directly connected to a diffusion pump filled with silicone oil. Before introduction of the samples, the Dry-Ice trap of the vacuum line was thoroughly dried. This insured that only water from the samples was present in the vacuum line during removal of D_2O from the samples. The diffusion pump was isolated from the vacuum line during the removal of ice from the samples. Otherwise H_2O was introduced into the system through exchange of D_2O vapor with the hot diffusion pump oil.

Ice was removed from the samples by sublimation into the cold trap using only the mechanical pump. After about 90 min the ice was removed, and the pressure in the vacuum chamber (as measured by a Pirani gauge) dropped. Removal of the last traces of D_2O from the samples was accomplished by use of the diffusion pump.

After about 2-hr high vacuum drying, thoroughly dry CO_2 was passed into the vacuum chamber. The CO_2 must be dry since any water introduced upon breaking the vacuum will exchange with labile protein side-chain deuterium atoms, and a low value for the degree of exchange will result. The CO_2 was dried by passing it through silica gel, several U tubes filled with anhydrous magnesium perchlorate, and two Dry-Ice–alcohol cold traps. About 45 min were allowed for passage of enough CO_2 into the vacuum chamber for attainment of atmospheric pressure. Then the samples were removed and quickly capped with dry caps. The presence of CO_2 minimizes passage of water vapor from the air into the sample flask by means of convection after removal and before capping of the sample.

NaOH (500 μl of 0.08 M) was then added to each sample. After the samples had dissolved they were allowed to stand overnight and were then heated to 80° for a time long enough to give turbidity or precipitation. In some of the samples, this procedure was reversed. The D_2O containing water was then isolated by cyrosublimation into an acceptor tube using Linderstrom-Lang's procedure (Krause and Linderstrøm-Lang, 1955).

The amount of D_2O in the water samples was determined by infrared analysis. Standards were made up by weight and the number of units of D_2O present was calculated (Hvidt *et al.*, 1954). Optical densities of the standards and unknowns were determined using a Perkin-Elmer Model 221 double-beam infrared spectrometer and CaF_2 cells of 0.126-mm path length. A separate calibration curve was made each time samples were analyzed. Analytical measurements were made at 2512 cm^{-1}. During the measurements H_2O *vs.* H_2O blanks were frequently measured and optical densities for all D_2O containing samples were corrected for drifts in the blank readings. Generally four standards were used for a calibration curve, and Beer's law was obeyed.

The number of hydrogen atoms which have been exchanged for deuterium per molecule of bovine plasma albumin can be calculated from the equation (Krause and Linderstrøm-Lang, 1955)

$$h_{measd} = \frac{110.8}{C_s} \frac{U_f}{1067} \quad (2)$$

where C_s is the number of millimoles of bovine plasma albumin (mol wt 66,000) present per milliliter of water in the back exchange solution (the solution formed after H_2O has been added to the deuterated protein), and U_f is the number of units of D_2O present in the water sample. The fact that a smaller concentration of protein was present in the back-exchange solution than in the forward exchange solution was taken into account in the calculation of C_s.

The accuracy of the method was first checked using glycine. Duplicate determinations gave results which were both within 1% of the theoretical value (three exchangeable hydrogens

TABLE I: Amino Acid Analysis of Bovine Plasma Mercaptalbumin.[a]

Amino Acid	No./ Molecule	Side-Chain Hydrogens	Peptide Hydrogens
Glycine	16	0	16
Alanine	46	0	46
Valine	36–37	0	36–37
Leucine	62	0	62
Isoleucine	14	0	14
Proline	30	0	0
Phenylalanine	27	0	27
Tyrosine	20	20	20
Tryptophan	2	2	2
Serine	26	26	26
Threonine	34	34	34
Half-cystine	35	0	35
Cysteine	1	1	1
Methionine	4	0	4
Arginine	22–23	110–115	22–23
Histidine	17	34	17
Lysine	62	186	62
Aspartic acid	54	54	54
Glutamic acid	77–78	77–78	77–78
Amide NH_2	32–33	(32–33)	0
Total		576–583	555–558

[a] Data of Spahr and Edsall (1964) for mol wt 66,000.

per molecule of glycine). A more stringent test of the method is to see if the theoretical number of hydrogens are obtained for complete exchange of a protein. Certain of the side-chain hydrogens in a protein could rapidly exchange with any H_2O vapor present in the system with the result that a low value for the number of exchangeable hydrogens in the protein would be obtained. Our results with bovine plasma albumin at pD 2.61 will serve as a test for the method. At this value of pD the bovine plasma albumin molecule should have a reasonably unfolded structure and the theoretical number of exchangeable hydrogens would be expected. From the amino acid analysis data (Table I) we can estimate that there are about 1137 exchangeable hydrogens/bovine plasma albumin molecule (mol wt 66,000). Our results at pD 2.61 and after 20 hr, at which time the extent of exchange has reached a constant value, gave an average value of 1099 for the number of hydrogens which exchanged. To this number should be added approximately four hydrogens corresponding to carboxylate groups not protonated at this value of pD. The experimental result is within 3% of the theoretical value. Considering uncertainties in amino acid analysis and the possibility that small amounts of nonprotein material might have been present in the lyophilized bovine plasma albumin, we can conclude that the agreement is satisfactory.

Since D_2O is removed from samples placed in a vacuum chamber, there is less chance that the temperature of the ice can become high enough for appreciable exchange to occur between ice and protein during the removal of D_2O from the protein. Some measurements were made of the temperature of ice during its sublimation in the vacuum chamber and during its sublimation using the conventional Linderstrøm-Lang donor–acceptor tube technique (Krause and Linderstrøm-Lang, 1955) where the vacuum pump is cut off from the system after evacuation and the outside of the donor tube is exposed to the atmosphere.

An iron–constantan thermocouple was inserted into the vacuum chamber and sealed with Apiezon W wax. At the start of the experiment the thermocouple junction was placed in a pear-shaped flask along with 500 μl of water. The water was frozen in the usual way, the vacuum chamber closed, and sublimation of the water carried out as described above. During the time that enough ice remained in the flask to cover the thermocouple junction (~1 hr), the recorded temperature remained constant at about $-31°$. The experiment was repeated with four such flasks each containing 500 μl of water. This time the ice in the flask containing the thermocouple maintained a temperature of $-24°$.

In order to measure the temperature of the ice when using the Linderstrøm-Lang technique, a thermocouple was inserted into a pear-shaped donor flask containing 500 μl of water. The donor flask was connected to a Y tube (Krause and Linderstrøm-Lang, 1955) along with an acceptor flask. The water in the donor flask was frozen by means of a Dry-Ice–alcohol bath and the system evacuated for 3 min. After evacuation the cold bath was placed around the acceptor tube and sublimation allowed to proceed. The ice temperature during the sublimation was about $-13°$. The new method thus reduces possible errors due to exchange in ice since the ice temperature is appreciably less when the vacuum chamber is used.

Errors due to possible exchange between dry protein and water vapor during the course of lyophilization are also reduced since the removal of ice is accomplished by continuous pumping, and the water vapor pressure is therefore lower.

The measurements reported here are for slow exchange since no experimental data were collected earlier than about 15 min after the start of exchange. Since experimental errors of the type discussed above are most serious for rapid exchange (Hallaway and Benson, 1965), we feel that any systematic errors in our results are negligible.

The use of high vacuum in drying the partially deuterated protein eliminates the necessity of heating the protein to 60° to remove the last traces of D_2O (Krause and Linderstrøm-Lang, 1955). The protein is not heated before an exchange run, so danger of denaturation before exchange is eliminated.

The method described here is more suited for measuring the exchange of an ordinary protein with D_2O rather than the exchange of a predeuterated protein with H_2O as in the conventional Linderstrøm-Lang method. It therefore may be used in conjunction with measurements of H–D exchange by means of changes in infrared transmission taking place when an ordinary protein is dissolved in D_2O (Blout *et al.*, 1961; Hvidt, 1963). Although it is more rapid, the infrared method is not as accurate as the method described here. Also the infrared method only approximates measurement of the number of hydrogens exchanged, whereas the present method can determine this number quite accurately.

Results

The amino acid analysis of bovine plasma albumin and the total number of exchangeable hydrogens are given in Table I. The data are taken from the work of Spahr and Edsall (1964).

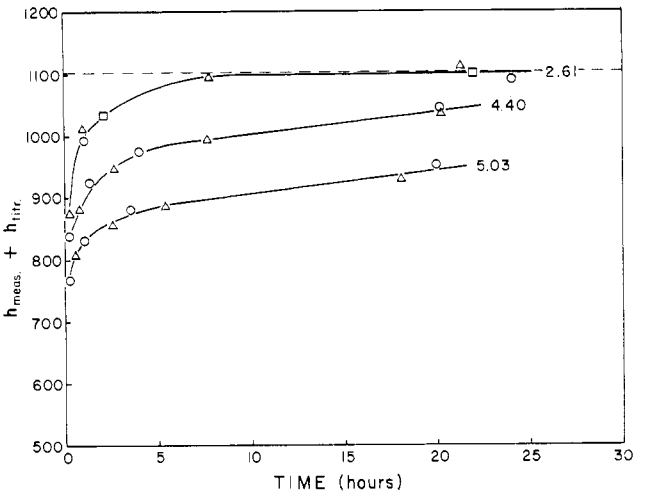


FIGURE 1: Hydrogen-exchange data ($h_{measd} + h_{titr}$) *vs.* time for bovine plasma albumin (temperature 30.0°, μ = 0.15) at various values of pD as indicated. Values of h_{titr} are: 4 (pD 2.61), 56 (pD 4.40), and 81 (pD 5.03). Different symbols represent points from separate experiments.

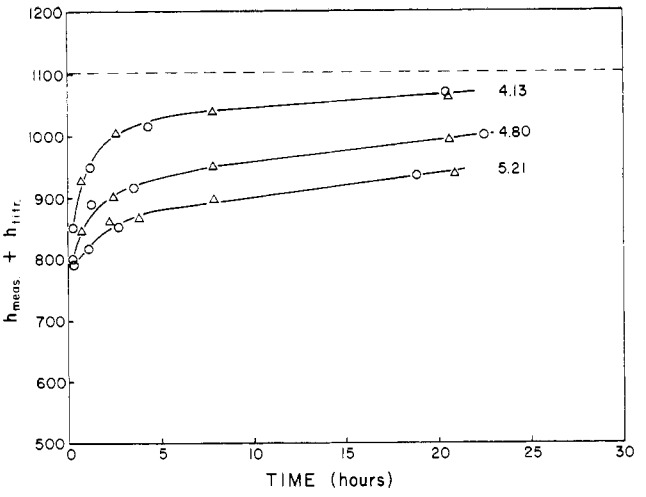


FIGURE 2: Hydrogen-exchange data ($h_{measd} + h_{titr}$) *vs.* time for bovine plasma albumin (temperature 30.0°, μ = 0.15) at various values of pD as indicated. Values of h_{titr} are: 46 (pD 4.13), 75 (pD 4.80), and 85 (pD 5.21). Different symbols represent points from separate experiments.

Generally exchangeable hydrogens can be divided into two classes: side-chain hydrogens which are generally assumed to exchange at a rate too fast for measurement using the present method and peptide hydrogens whose rate of exchange varies but can be quite slow. The theoretical number of hydrogens corresponding to complete exchange at any pH can be obtained as the sum of the side-chain and peptide hydrogens less the number of protons removed from the completely protonated protein, h_{titr}. We have used the data of Tanford *et al.* (1955a) in order to obtain h_{titr} and have assumed that values for h_{titr} at any pH in H_2O are the same as those for corresponding values of pD_{app} in D_2O. The approximate validity of this assumption has been demonstrated by Vijai and Foster (1967).

Experimental results are presented in Figures 1–3 where values of ($h_{measd} + h_{titr}$) are plotted at various pD values as a function of time. At least two separate runs were made at each value of pD, and the points from separate runs are distinguished to give an idea of the reproducibility of the method. Nearly all of the points at all values of pD fall within 1% of the smooth curves drawn through the points, and no point in any of the runs has an error of more than 3%.

If we assume that exchange at pD 2.61 (Figure 1) goes to completion, then the experimental value for the total number of exchangeable hydrogens, h, is given by

$$h = (h_{measd} + h_{titr})_{pD\ 2.61} = 1103 \qquad (3)$$

This value is indicated by the dotted line in Figures 1–3. The number of hydrogens not exchanged at any value of pD, h_i, is given by

$$h_i = h - (h_{measd} + h_{titr})_{pD} \qquad (4)$$

If we assume that all of the hydrogens in a protein can exchange at different rates and independently of each other, then

$$h_i = \sum_m n_m e^{-k_m t} \qquad (5)$$

where there are m classes of exchanging hydrogens each of which is characterized by n_m hydrogens and a first-order rate constant for exchange k_m.

Values of h_i were calculated from points taken from the smooth curves drawn through the experimental points in Figures 1–3. Semilogarithmic plots of h_i against t were then made for each pD value. Examination of these curves indicated that nearly all of them could be resolved into two well defined linear exchange curves characterized by different values of n_m and k_m. Values for n_m, k_m, and $t_{1/2,m}$, the half-time for each exchanging class, are given in Table II.

The mechanism of hydrogen exchange in proteins has been discussed by Hvidt (1964), Hvidt and Nielsen (1966), and W. P. Bryan (to be published). The results cited here appear to be

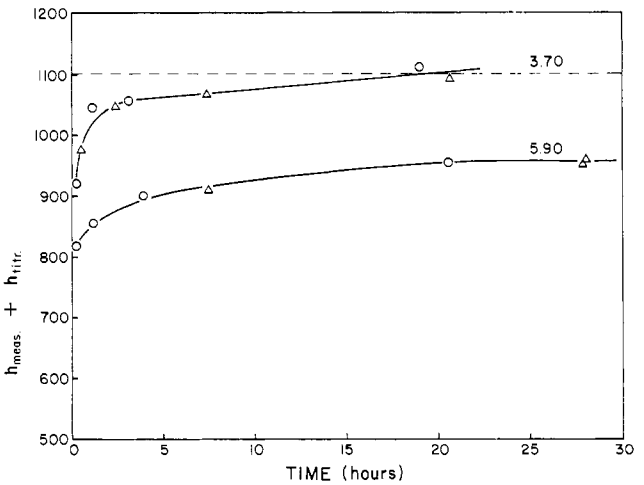


FIGURE 3: Hydrogen-exchange data ($h_{measd} + h_{titr}$) *vs.* time for bovine plasma albumin (temperature 30.0°, μ = 0.15) at various values of pD as indicated. Values of h_{titr} are 26 (pD 3.70) and 99 (pD 5.90). Different symbols represent points from separate experiments.

TABLE II: Class Size, Rate Constants, and Half-Times for Exchange of Bovine Plasma Albumin at 30.0°, $\mu = 0.15$.

pD	n_m	$k_m \times 10^2$ (min^{-1})	$t_{1/2,m}$ (hr)	Σn_m
2.61	206	4.6	0.25	326
	120	0.52	2.2	
3.70	143	2.17	0.53	193
	50	0.140	8.2	
4.13	179	1.21	0.95	261
	82	0.068	17	
4.40	154	1.05	1.1	284
	130	0.061	19	
4.80	111	1.05	1.1	302
	191	0.0437	26.3	
5.03	92	1.15	1.0	302
	210	0.0329	35.0	
5.21	80	0.82	1.4	319
	239	0.0311	37.0	
5.90	~29	~6	~0.2	291
	118	0.274	4.2	
	144	~0	~∞	

best explained by means of the Linderstrøm-Lang (1955) mechanism. In this mechanism exchange is thought to occur through some intermediate structure I which is produced by means of a transconformational change of a stable or native structure N.

$$\begin{aligned} \mathrm{N} + \mathrm{D_2O} &\underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{I(D_2O)} \\ \mathrm{I(D_2O)} &\xrightarrow{k_3} \text{exchange} \end{aligned} \tag{6}$$

N and I generally refer to a region of the molecule of size n_m characterized by a specific value of k_m. The exchanging hydrogens in I are generally assumed to be completely exposed to water. Therefore, understanding of the step characterized by k_3 can be obtained from the study of completely exposed model substances.

If we assume a steady-state concentration of $\mathrm{I(D_2O)}$, we have

$$k_m = \frac{k_1 k_3}{k_2 + k_3} \tag{7}$$

Two limiting cases are of interest. If $k_3 \gg k_2$

$$k_m = k_1 \tag{8}$$

The rate of exchange is given by the rate of the transconformational opening of the protein structure. Such exchange has been designated EX_1 (Hvidt and Nielsen, 1966) or EU_1 (W. P. Bryan, to be published). If $k_2 \gg k_3$

$$k_m = \frac{k_1}{k_2} k_3 \tag{9}$$

In this case $\mathrm{I(D_2O)}$ is formed and removed many times before exchange can occur. This has been designated as EX_2 or EU_2.

In general these two cases can be distinguished since studies of model compounds (Hvidt and Nielsen, 1966; Leichtling and Klotz, 1966) show that k_3 is approximately given by

$$k_3 = k_0 + k_{\mathrm{D}}(\mathrm{D^+}) + k_{\mathrm{OD}}(\mathrm{OD^-}) \tag{10}$$

For a protein k_3 would be expected to have a minimum value at a pD of about 3.0 or below. The value of k_0 is generally small. Therefore for most pD values of interest, $k_3 \cong k_{\mathrm{OD}}(\mathrm{OD^-})$. Thus for the EU_1 case k_m should be pD independent if k_1 is, and for the EU_2 case k_m should depend upon $(\mathrm{OD^-})$ provided k_1/k_2 is pD independent.

It is convenient to consider the results given in Table II in terms of three different regions of pD. The results at pD 5.90 correspond to bovine plasma albumin in its native or N form. Between pD values of approximately 5.0 and 4.0 the well-known N–F isomerization occurs (Foster *et al.*, 1965). Our results at pD values from 4.13 to 5.21 can be considered as lying in the region of N–F isomerization. Below pD values of about 4.0 a reversible molecular expansion of the bovine plasma albumin molecule occurs. The results at pD values of 2.61 and 3.70 can be considered as pertaining to expanded bovine plasma albumin. The experiments reported here were all carried out at an ionic strength of 0.15 in an effort to ensure that, upon lowering the pD, the onset of molecular expansion occurred after the disappearance of the N form (Tanford *et al.*, 1955b).

Since structural changes are occurring in the bovine plasma albumin molecule over the pD range of our measurements, mechanistic interpretation becomes difficult. We shall focus on the simplest way in which our results can be explained. Consider the results in the N–F transition region at pD 4.13–5.21. Two classes of slowly exchanging peptide hydrogens are observed. Other peptide hydrogens exchange too rapidly to be seen in our experiments. The pD in the N–F region is high enough so that a roughly first-order dependence of k_m on $(\mathrm{OD^-})$ would be expected if the exchange mechanism were of the EU_2 type. Examination of the results in Table II shows that k_m for the faster observed class is approximately constant and k_m for the slower observed class decreases from 7×10^{-4} to 3×10^{-4} min^{-1} as pD increases from 4.1 to 5.2. Such behavior is consistent with an EU_1 type of mechanism with a slight pD dependence of k_1. We shall assume that the EU_1 mechanism also holds at pD 5.90 since the higher the value of pD the greater the likelihood of such a mechanism (W. P. Bryan, to be published). This is also consistent with the results of Benson *et al.* (1964) who observed EU_1 like behavior at 0° in this pH region. Values of k_m in the region of molecular expansion (pD values of 3.70 and 2.61) increase somewhat as pD is lowered. It is not possible to definitely choose mechanisms here. The mechanism might be EU_1 with a k_1 value which increases with decreasing pD due to molecular expansion. Alternatively the mechanism might be EU_2, and the increase in k_m values might be explained by the beginning of $(\mathrm{D^+})$ catalysis (eq 10).

It is interesting to compare values of h_i at a time long enough after the start of exchange so that exchange in the region of molecular expansion has gone to completion. Since exchange outside of this region is assumed to be by the EU_1 mechanism, a plot of h_i *vs.* pD should give an indication of the number of nonexchanged hydrogens which correspond to structural re-

gions in which exchange is slow. A plot of h_i *vs.* pD after 20 hr of exchange is shown in Figure 4. There appears to be a transition in h_i from a value of 0 to about 165 as pD goes from 3.8 to 5.2. This transition in h_i clearly corresponds to the N–F isomerization region for bovine plasma albumin. Similar results obtained by following exchange by an infrared method were reported by Foster *et al.* (1965).

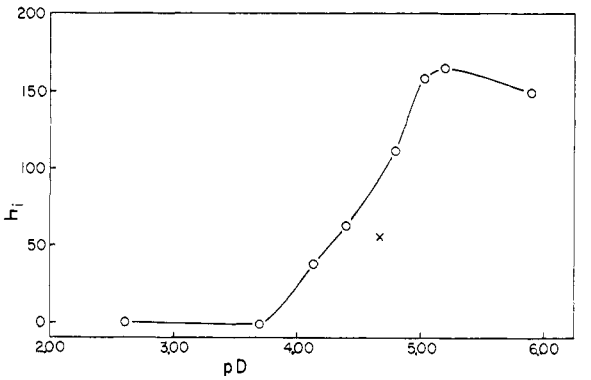


FIGURE 4: Number of hydrogens not exchanged after 20-hr exchange, h_i, *vs.* pD. The x indicates the value of h_i after 10-hr exchange at pD 4.13 (see text).

Discussion

As the pH of a solution of bovine plasma albumin is lowered from the region of its isoelectric point, a very interesting isomerization occurs. This N–F transition has been much studied, particularly by Foster and coworkers. Earlier work has been summarized by Foster (1960). In the pH region of the N–F transition two major electrophoretic components can be observed. Above a pH of about 4.5 only the N form is present and below a pH of about 3.5 only F form is present. At lower pH values a molecular expansion takes place upon lowering of the pH. This expansion is ionic strength dependent and is well separated from the N–F isomerization at $\mu = 0.15$. In D_2O the N–F transition occurs in the pD region of about 4.0–5.0.

The nature of the N–F transformation has been explained by Sogami and Foster (1963) and Foster *et al.* (1965). In order to reconcile electrophoretic separation of the two forms with rapid attainment of the N–F equilibrium upon adjustment of pH, these investigators postulated that a microheterogeneity exists in bovine plasma albumin. Native bovine plasma albumin is assumed to consist of similar species which are only slowly interconvertible. Each of these native (N) species undergoes a sharp and fast transformation to a corresponding F species over some narrow range of pH in the region of the over-all N–F isomerization. Thus, when the pH of a solution of isoelectric bovine plasma albumin is lowered to somewhere within the 3.5–4.5 region, certain of the N species are rapidly converted to the corresponding F species, but the pH is still above the pH intervals for the transformation of the remaining N species. The electrophoretic separation is assumed to be a separation of N forms stable at the pH of electrophoresis from F forms stable at the same pH.

A number of experimental studies are consistent with this explanation. Thus, electrophoretic studies (Sogami and Foster, 1963), electrophoretic, hydrogen exchange, solubility studies (Foster *et al.*, 1965), fractionation by solubility studies (Peterson and Foster, 1965a), thermal denaturation studies (Stokrova and Sponar, 1963), optical rotation, spectral, and titration studies (Petersen and Foster, 1965b) are all in accord with the concept of microheterogeneity. Recently some doubt has been cast on the reality of this microheterogeneity when all fatty acid impurities are removed from bovine plasma albumin (McMenamy and Lee, 1967). McMenamy and Lee have shown that bovine plasma albumin preparations defatted by their procedure exhibit much sharper N–F transitions than ordinary or conventionally defatted bovine plasma albumin. However, Sogami and Foster (1968) have indicated that bovine plasma albumin defatted by the charcoal method of Chen (1967) still shows microheterogeneity. Also if the N–F isomerization is rapid, the shape of the solubility curves of defatted preparations obtained by McMenamy and Lee still support the microheterogeneity model (Foster *et al.*, 1965).

Interesting changes in the properties of bovine plasma albumin occur upon isomerization. For example, conversion of N into F is accompanied by loss of some helical structure (Leonard and Foster, 1961a; Sogami and Foster, 1968). Conversion of N into F is accompanied by the unmasking of about 40 carboxyl groups (Vijai and Foster, 1967). When N is converted into F, ability to bind large quantities of hydrocarbon is lost (Wishnia and Pinder, 1964). Solvent perturbation studies (Herskovits and Laskowski, 1962) indicate further exposure of aromatic chromophores upon N–F isomerization. Under certain conditions, but not those of the present study, the N–F isomerization occurs in two steps upon lowering of the pH (Leonard and Foster, 1961a). Leonard and Foster (1961b) have also pointed out similarities between the N–F transformation and the two-step unfolding of bovine plasma albumin upon binding of large numbers of detergent molecules.

Our data at pD 5.90 correspond to bovine plasma albumin in its N form. After 20-hr exchange h_i has a value of 150. This is in good agreement with the value of about 145 for the number of hydrogens not exchanged after 24 hr (25°, pH ~ 5) obtained by Benson *et al.* (1964). There is some question as to whether the slowly exchanging hydrogens of native bovine plasma albumin are all peptide hydrogens. Masked amino groups appear to be present in the N form (Vijai and Foster, 1967) and these could exchange slowly. We have no evidence which could rule out such a contribution to slow exchange.

Data at pD values of 2.61 and 3.70 correspond to exchange of bovine plasma albumin when it is in an expanded form. Half-times for exchange of random coil peptide hydrogens taken from the data of Bryan and Nielsen (1960) for poly-DL-alanine are ~3 min at pD 3.70 and even less (~1 min) at pD 2.61. Reference to Table II shows that there is a great deal of structure still present in bovine plasma albumin under these conditions since half-times for exchange are much greater than for random coil peptide hydrogens. This result is not surprising since previous work has also indicated some compact globular structure of bovine plasma albumin at low pH. Evidence for such structure comes from studies of optical rotation (Sogami and Foster, 1968), low angle X-ray scattering (Luzzatti *et al.*, 1961), pepsin hydrolysis (Weber and Young, 1964), and solvent perturbation spectroscopy (Herskovits and Sorensen, 1968).

The numbers of hydrogens which exchange slowly at pD 2.61 are appreciably greater than the numbers which exchange slowly at pD 3.70. This is true despite the fact that the rates of

exchange are faster at the lower value of pD. A possible explanation is that some of the hydrogens observed to exchange slowly at pD 2.61 are associated with lysine side chains. Observations of the rates of exchange of ammonium (Emerson *et al.*, 1960) and methylammonium ions (Grunwald *et al.*, 1960) in acid solution indicate that at pD values of ~2 and below the rate of exchange of lysine side-chains hydrogens should be of the same order of magnitude as the rate of exchange of random coil peptide hydrogens. This is because exchange of ammonium or methylammonium ions is not acid catalyzed. Another possible explanation for the greater number of slowly exchanging hydrogens at pD 2.61 is the tendency for the protein to associate at low values of pD and the high ionic strength used in this study (Williams and Foster, 1960).

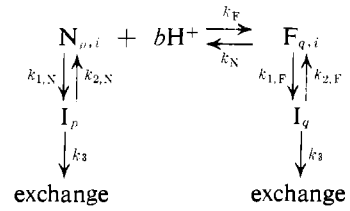
In considering the exchange results in the region of the N–F isomerization we shall assume that the exchange curve at pD 4.13 essentially corresponds to exchange of the F form of bovine plasma albumin and that the exchange curve at pD 5.20 essentially corresponds to exchange of the N form. In agreement with the conclusion of Foster *et al.* (1965), we can show that our results are compatible with microheterogeneity but not the presence of only one N and one F form of bovine plasma albumin. Let us assume that there is a rapid equilibrium between only one N and one F form of bovine plasma albumin.

$$\mathrm{N} + a\mathrm{H}^+ \underset{k_{\mathrm{N}}}{\overset{k_{\mathrm{F}}}{\rightleftharpoons}} \mathrm{F} \qquad (11)$$

In nondefatted bovine plasma albumin a should be ~3. Further, let us for the moment assume that exchange of the slowly exchanging hydrogens can only occur through the F form. Now consider the 20-hr values of h_i (Figure 4). At the midpoint of the isomerization region, pD 4.67, we would expect each molecule to spend about half of its time in the N form. Therefore at pD 4.67 h_i at 20-hr exchange should correspond to h_i at 10-hr exchange at pD 4.13. The value of h_i at 10-hr exchange at pD 4.13 is indicated by an X in Figure 4. It falls below the curve. If we also allow some exchange through the N form to occur, the point predicted for exchange at pD 4.67 would be even lower. Thus, the experimental curve of h_i *vs.* pD is not compatible with this model.

If the equilibrium indicated in eq 11 is assumed very slow and exchange only occurs through the F form, then the lower values of k_m given in Table II might be approximately equal to $k_{\mathrm{F}}(\mathrm{H}^+)^a$, or at least be strongly pH dependent. Reference to Table II shows that the lower value of k_m has a very slight pD dependence in the isomerization region. Therefore, irrespective of whether the equilibrium of eq 11 is fast or slow, the hydrogen-exchange results are not compatible with single N and F forms.

The hydrogen-exchange results can be explained in terms of the microheterogeneity model and the Linderstrøm-Lang mechanism. Consider exchange as occurring according to eq 12. $\mathrm{N}_{p,i}$ represents a region of the ith microheterogeneous type of N molecule of a size corresponding to p-exchanging hydrogens. $\mathrm{F}_{q,i}$ represents a similar region of an F molecule corresponding to q-exchanging hydrogens. The coefficient b corresponds to the pH dependence of the N–F transition for a single microheterogeneous species. The value of b is high



(12)

enough so that the transition is extremely sharp; or, put in another way, anywhere in the isomerization region a particular species i will always be either almost completely N_i or F_i. The equilibrium characterized by $k_{\mathrm{F}}/k_{\mathrm{N}}$ is rapid. Exchange of an N region occurs through I_p and exchange of an F region occurs through I_q. Since the results indicate two classes of slowly exchanging hydrogens at both pD 4.13 and pD 5.21; there must be at least two N regions, $\mathrm{N}_{p,i}$ and $\mathrm{N}_{p',i}$, and two F regions, $\mathrm{F}_{q,i}$ and $\mathrm{F}_{q',i}$. From Table II we can assign the approximate values: $p = 80, p' = 239, q = 179$, and $q' = 82$.

For sake of simplicity we will assume that the rate of exchange of N_i or F_i is independent of i. The question arises as to whether the previously assumed EU_1 mechanism is compatible with eq 12 or, in other words, are $k_{m,\mathrm{N}}$, $k_{m',\mathrm{N}}$, $k_{m,\mathrm{F}}$, and $k_{m',\mathrm{F}}$ pD independent, where the k values are the measured rate constants for exchange of N and F forms. The exchange at pD 4.80 can be used to test this point. The larger value of k_m observed here should be dominated by $k_{m,\mathrm{F}}$, and the smaller value of k_m should be dominated by $k_{m,'\mathrm{N}}$. Examination of Table II shows that there is no indication that $k_{m,\mathrm{F}}$ or $k_{m',\mathrm{N}}$ show a pD dependence in conformity with an EU_2 mechanism. We will therefore assume that an EU_1 mechanism holds and that all four k values are independent of pD. We therefore assign the approximate values: $k_{m,\mathrm{N}} = k_{1,\mathrm{N}} = 0.8 \times 10^{-2}$ min^{-1}; $k_{m',\mathrm{N}} = k_{1',\mathrm{N}} = 3 \times 10^{-4}$ min^{-1}; $k_{m,\mathrm{F}} = k_{1,\mathrm{F}} = 1 \times 10^{-2}$ min^{-1}; $k_{m',\mathrm{F}} = k_{1',\mathrm{F}} = 7 \times 10^{-4}$ min^{-1}. The precision of our data is such that the pairs of constants $k_{1,\mathrm{N}}$, $k_{1,\mathrm{F}}$, and $k_{1',\mathrm{N}}$, $k_{1',\mathrm{F}}$ cannot be resolved in the isomerization region.

The analysis leads to a picture of the N–F isomerization of bovine plasma albumin in which both N and F forms have two regions of slowly exchanging hydrogens. When isomerization of an N form occurs, the more rapidly exchanging region grows at the expense of the more slowly exchanging region. The rate of opening of both of these regions is only slightly affected by the isomerization reaction.

We would like to emphasize that, since exchange occurs through an I form, the size of an exchanging region need have no correspondence to the size of structurally similar regions in the native protein. Exchange which occurs by the Linderstrøm-Lang mechanism cannot, in general, be used to draw conclusions about the size of structurally similar regions in a native protein (W. P. Bryan, to be published). With this disclaimer we can, however, present a speculative correlation of our results with conclusions from optical rotation measurements. Sogami and Foster (1968) have estimated helical content of bovine plasma albumin by means of optical rotatory dispersion studies. Their average estimate for untreated bovine plasma albumin at neutral pH is 54%, and for bovine plasma albumin in the F form (pH 3.6–3.9) they estimate 47%. These values can be compared with Σn_m values from Table II divided by the total number of peptide hydrogens per bovine plasma albumin molecule: 557. Thus at pD 5.21 we obtain 57% helix and at pD 4.13 (F form) 47% helix. We should note, however, that Benson *et al.* (1964) were unable to obtain a correlation

between slowly exchanging hydrogens and per cent helix in their work.

Nevertheless our agreement appears good enough to tempt us to further speculation. Thus, we can suggest that if these slowly exchanging hydrogens can be identified with helical regions, then bovine plasma albumin (both N and F forms) should have two helical regions which open in different ways. Under certain conditions, such as the presence of SCN^- or ClO_4^-, the N–F isomerization takes place in two steps (Leonard and Foster, 1961a). These two steps might correspond to the isomerization of each helical region separately. A similar two-step process occurs upon the binding of detergents to bovine plasma albumin at neutral pH (Leonard and Foster, 1961b). Studies of the enzymatic hydrolysis of bovine plasma albumin at low pH (Weber and Young, 1964) or at high detergent concentration (Adkins and Foster, 1965) indicate that bovine plasma albumin could be composed of two globular regions which might be related to the two classes of slowly exchanging hydrogens observed here.

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