Deuterium–Hydrogen Exchange of Muscle Proteins*

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ABSTRACT: At apparent pH values of 6.3 the percentage of hard-to-exchange peptide hydrogens for the proteins light meromyosin fraction I, tropomyosin, actin, myosin, and heavy meromyosin bore little apparent relationship to their reported helical contents. Heavy meromyosin contained the highest percentage of hard-to-exchange peptide hydrogens. Increasing pH increased the exchange rate for all of these proteins. The effect of pH on the exchange rates of the highly helical proteins, light meromyosin fraction I, and tropomyosin was more marked than for the proteins heavy meromyosin and myosin. From the dissimilarity of the pH dependence of exchange of heavy meromyosin it is proposed that some conformation other than the α helix is partly responsible for the higher percentage of unexchanged peptide hydrogens in this protein. Sodium dodecyl sulfate increased the exchange rates for all the proteins but also altered considerably the physical properties of the studied proteins, heavy meromyosin and light meromyosin fraction I.

The origin of the hydrogens of proteins which exchange slowly with deuterium has still not been completely resolved. Linderstrøm-Lang (1955) proposed that in insulin these hydrogens were in firmly locked helical regions. More recently for α-keratin, Fraser and MacRae (1959) attributed the stability of certain hydrogens to exchange to its α-helical configuration, cyclic nature, and to side chain interactions. Blout *et al.* (1961), from a study by the infrared method of the exchange characteristics of several proteins, postulated that, in addition to the hydrogens of the α-helical regions, there may be other regions containing hard-to-exchange amide hydrogens. Leonard *et al.* (1965) suggested that the "nonexchangeable" hydrogens of native albumin result from masking by the tertiary structure and not from the helical structure *per se*.

In an attempt to identify the origin of these hard-to-exchange hydrogens, a study of the deuterium–hydrogen exchange properties of some of the muscle proteins was undertaken. Of these proteins, light meromyosin fraction I and tropomyosin have b_0 values indicative of almost 100% α helix (Cohen and Szent-Györgyi, 1957). More recent determinations in the ultraviolet, however, have yielded slightly lower values of approximately 87% (Shechter and Blout, 1964). Myosin and heavy meromyosin have intermediate helical contents while the value for actin has been estimated as low as 8% (Kay, 1958).

In view of the studies by Hvidt (1964) and Benson *et al.* (1964) concerning the *p*H dependence of the deuterium–hydrogen exchange, it was of interest to investigate the effect of varying *p*H on the exchange properties of these proteins, bearing in mind their wide divergence of helical content. In addition the effect of sodium dodecyl sulfate on the exchange rates of these proteins was investigated. This detergent was used by Hvidt *et al.* (1963) to obtain completely deuterated samples of yeast alcohol dehydrogenase and by Di Sabato and Ottesen (1965) for lactic acid dehydrogenase. In these studies the infrared method was used which measures only the exchange of the peptide hydrogens.

Materials and Methods

Deuterium oxide (99.76% D_2O) was purchased from Bio-Rad Laboratories, California. Sodium dodecyl sulfate (SDS),[1] obtained from Matheson Coleman and Bell, New Jersey, was recrystallized twice from methanol and once from water. All other chemicals used were reagent grade.

Protein Preparations. The rabbit *longissimus dorsi* muscle was the source of the proteins used in this study. Myosin A was prepared according to a modified Szent-Györgyi (1951) method. The method of Lowey and Cohen (1962) was used for the preparation of heavy meromyosin (HMM), and light meromyosin fraction I (LMM Fr. I) was isolated following the procedure of Szent-Györgi *et al.* (1960). Crystalline tropomyosin was obtained by the method described by Bailey (1948). G-Actin was prepared by the Carsten and Mommaerts (1963) method. Actin was lyophilized in the G form although during deuteration a certain amount of polymerization did occur.

Protein concentrations were determined using the micro-Kjeldahl technique, assuming a nitrogen content

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; HMM, heavy meromyosin; LMM Fr. I, light meromyosin fraction I.

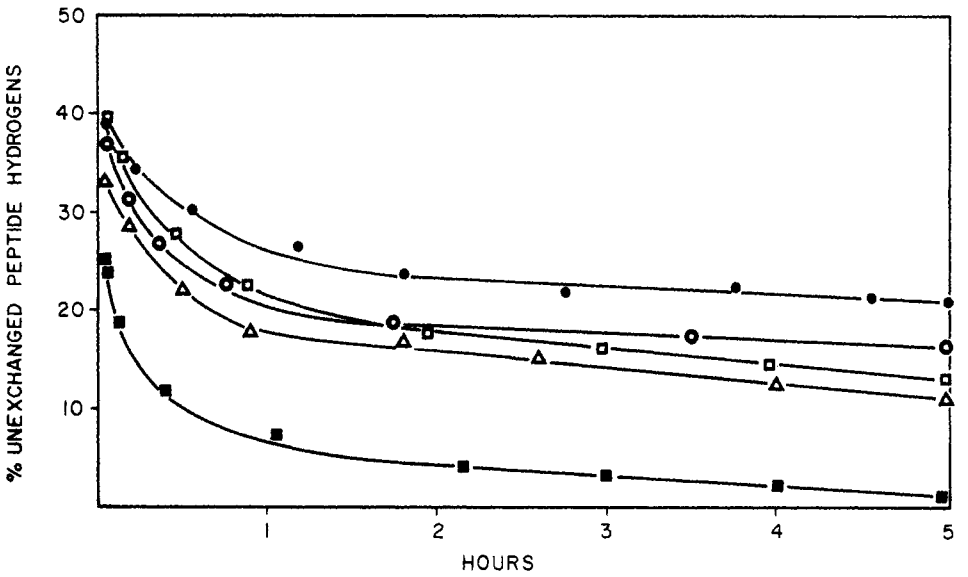

FIGURE 1: The deuterium-hydrogen exchange rates of the peptide hydrogens of the proteins HMM (●), myosin (○), LMM Fr. I (□), and actin (△) at apparent pH 6.3, and of the protomyosins (■) at apparent pH 6.7.

of 16.2% (Lowey and Kucera, 1964). Prior to deuteration the proteins in normal solvent mixtures were lyophilized in the presence of sucrose. After the addition of D_2O to the lyophilized protein the sucrose concentration was 15%. The protein concentrations varied between 1 and 2%. The *p*H range of the samples was attained by the addition of a mixture of KH_2PO_4 and K_2HPO_4 to a final phosphate concentration of 0.05 M after solution in D_2O.

Infrared Spectral Determinations. The method was essentially that described by Blout *et al.* (1961) and Hvidt (1963). Infrared spectra were determined with a Perkin-Elmer double-beam spectrometer (Model 21) using a sodium chloride prism and calcium fluoride cells (0.1 mm path length). The starting time for deuteration was taken upon addition of D_2O to the lyophilized protein. Times given in the text were those when scanning began. Spectra were determined between 1786 cm^{-1} and 1515 cm^{-1}. The time to scan this frequency range was approximately 1 min. Longer scan times were avoided owing to heating of the sample in the radiation beam. Between runs the cells were removed from the spectrometer and maintained at 25° in a dry atmosphere. Deuteration was followed over a 5-hr period. The amide I band was initially recorded at approximately 1655 cm^{-1}. As deuteration of the protein proceeded, this bands hifted approximately 15 cm^{-1} to a longer wavelength. The amide I frequency was similar for all the muscle proteins studied. The amide II band was measured at 1544 cm^{-1}. The ratio of the amide absorbancies ($A_{\text{amide II}}/A_{\text{amide I}}$) was taken as a measure of the exchange as suggested by Blout *et al.* (1961). The transmittance (T), obtained from the spectrometer readings, was converted to absorbance (A) using the formula $A = -\log_{10} T$. The completely deuterated ratio for the samples was obtained after heating at 40° for 18 hr. Heating at 70° resulted in either precipitation of the proteins or gel formation unless detergent was present. The amide I absorbancy of such "denatured" samples was usually decreased although the amide II absorbancy remained unaltered. Prolonged heating at 40° occasionally resulted in the formation of a broad band at 1590 cm^{-1} (see Figure 5). This effect was most noticeable at pH values below 7. The band at 1590 cm^{-1} could correspond to the 1608 cm^{-1} band found by Doty *et al.* (1958) and attributed to the β-configuration. In samples where the 1590 cm^{-1} band was observed the exchange data were not used as it was found that the relationship between the amide I and the amide II bands was altered. A ratio of 0.46 was taken for the undeuterated protein (Hvidt, 1963). Preliminary studies of the infrared spectra of LMM Fr. I in chloroethanol also yielded this value. However, from this work the value obtained must be regarded as only approximate since it involved a correction of the amide II absorbancy, necessary because of the interference of the ionized carboxyl band. Results are expressed as the percentage of unexchanged hydrogens which was obtained as suggested by Hvidt (1963).

pH Determinations. The apparent pH of the protein solution in D_2O was determined on a Radiometer pH meter, Model TTT1, equipped with a GK 2021 B electrode. The pH figures given in the text refer to this value. The pD was not determined.

Optical Rotatory Dispersion. Measurements were made at 25° with a Rudolph Model 200 photoelectric spectropolarimeter equipped with an oscillating polarizer prism. A Xenon light source was used for the range 360–600 mμ. The parameter b_0 of the Moffit–Yang equation (1956) was obtained from the slope of the plot $[m']\lambda(\lambda^2 - \lambda_0^2)/\lambda_0^2$ against $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ as suggested by Urnes and Doty (1961). A value of 212 mμ

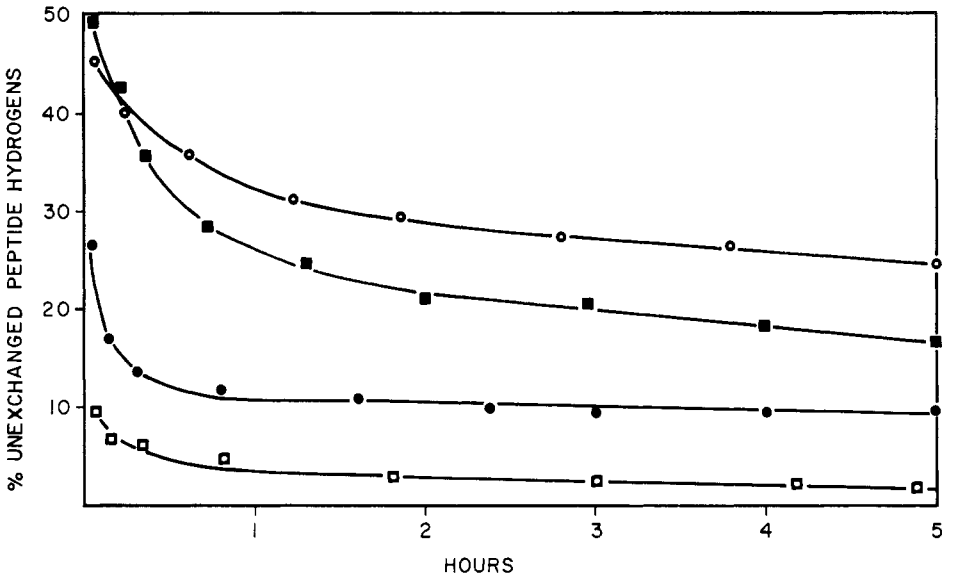

FIGURE 2: The deuterium-hydrogen exchange rates of the peptide hydrogens of LMM Fr. I at apparent pH values 6.05 (■) and 8.25 (□), and of HMM at apparent pH values of 5.96 (○) and 8.20 (●).

was chosen for the λ_0. A mean residue weight of 118 was used. Refractive indices were determined at 25° with a Zeiss Abbe refractometer (Model B) at 546 mμ.

Viscosity Measurements. Viscosity measurements were made in an Ostwald-type viscometer at 25 ± 0.01°. Chosen viscometers had water outflow times between 70 and 85 sec.

ATPase Measurements. ATPase activities were measured according to the procedure described by Perry (1955). The Gomori modification of the Fiske–Subbarow method (Gomori, 1942) was used to determine inorganic phosphorus.

Results

Lyophilization of the muscle proteins in the presence of sucrose yielded samples which were readily soluble and which on resolution exhibited the properties of the native protein. Mueller[2] observed that the ATPase activity of myosin and HMM was unaltered after lyophilization in normal solvent mixtures to which sucrose had been added. Our results indicated further that the optical rotation and viscosity characteristics of myosin, HMM, tropomyosin, and LMM Fr. I were also recoverable. Mean b_0 values corresponding to 56, 44, 98, and 92% α helix were obtained for myosin, HMM, LMM Fr. I, and tropomyosin, respectively. In the case of G-actin the protein after lyophilization retained the ability to polymerize.

The lyophilization and subsequent solubilization of HMM and tropomyosin was achieved in the absence of sucrose, although actin and myosin were rendered at least partially insoluble. Biro and Dekany (1964) point out that the condition of the samples before and during lyophilization is critical. It was assumed, therefore, that any conformational change occurring in the proteins during lyophilization was negligible and for the exchange studies the lyophilized proteins validly represented the native proteins.

The exchange of the peptide hydrogens for deuterium in the proteins myosin, HMM, LMM Fr. I, and actin is shown in Figure 1. For all of these proteins over 60% of the peptide hydrogens were exchanged in the first 3 min after solution in D_2O. The plateau region of the curves represented those hydrogens which exchanged slowly. As can be seen the percentage of slowly exchanging peptide hydrogens bears little apparent relationship to the reported helical content of these proteins. The percentage of unexchanged peptide hydrogens for HMM was higher at all points than for the more helical proteins, although the difference in the plateau region of the curves should be regarded as more significant. The exchange data for the protomyosins are also included in this figure. The protomyosins (Szent-Györgyi and Borbiro, 1956) approximated the random coil more closely than any of the other proteins, and exchange was found to be relatively rapid. At pH 6.3 the protomyosins were insoluble, hence the higher pH for the run reported here.

The hydrogen–deuterium exchange rates of myosin and HMM were found to be unaffected by ATP and Ca^{2+} or Mg^{2+} at concentrations of each up to 10 mM, although it should be stressed that this method may not reveal subtle conformational changes.

The effect of pH on the exchange rates of LMM Fr. I and HMM is shown in Figure 2. Again the HMM retained a higher percentage of slowly exchanging hydrogens compared with the LMM Fr. I, which after 5-hr exchange at pH 8.25 was almost completely

[2] H. Mueller, personal communication.

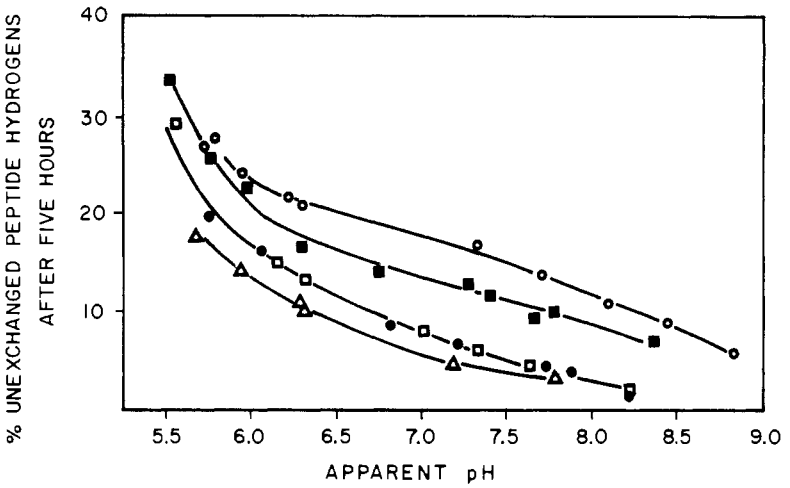

FIGURE 3: The effect of pH on the percentage of unexchanged peptide hydrogens remaining after deuteration for 5 hr for the proteins HMM (○), myosin (■), LMM Fr. I (●), tropomyosin (□), and actin (△).

deuterated. Tropomyosin in the absence of KCl behaved very similarly to LMM Fr. I.

The effect of pH on the deuterium–hydrogen exchange of the different proteins studied is shown in Figure 3. Here the percentage of unexchanged peptide hydrogens remaining after 5-hr exposure to D_2O was plotted *vs.* apparent pH. HMM at all points on the curve had the highest number of nonexchanged hydrogens. The curve for myosin approximated the expected curve assuming one LMM and one HMM per myosin molecule. Thus it would also appear that the relatively greater extent of deuteration of LMM Fr. I compared to myosin and HMM was not due to the presence of tryptic splits at arginine and lysine residues in the LMM Fr. I molecule. Also, tropomyosin and LMM Fr. I have similar helicity and both show the same pH dependence of exchange. The addition of KCl (to a final concentration of 0.5 M) to tropomyosin increased the 5-hr exchange value by approximately 2% throughout this pH range. The significance of this is not clear. It should be emphasized that the b_0 values of HMM, LMM Fr. I, myosin, and tropomyosin remained constant at the various pH values of these experiments. Optical rotatory dispersion measurements were also made on HMM and tropomyosin after 5 hr in D_2O and no effect of deuteration on b_0 could be detected. At neutral pH values the ATPase activity of myosin and HMM in D_2O showed the same slight decrease in activity, with time, exhibited by control solutions of these proteins in H_2O, both at 25°.

Samples of tropomyosin and HMM were lyophilized without sucrose and exchange curves were determined at different pH values. From these curves, which corresponded to those obtained in the presence of sucrose, it was concluded that sucrose had no detectable effect on the exchange rates for these proteins.

The effect of SDS on all the proteins studied was to reduce the percentage of hard-to-exchange hydrogens. The region of the HMM molecule that was relatively resistant to exchange was sensitive to this detergent. Figure 4 shows that at a detergent concentration of 1 mg/ml (3.5 mM) exchange was almost complete after 5 hr at pH 7.1. At a higher detergent concentration of 10 mg/ml the exchange was virtually complete after 30 min. At this latter concentration of detergent the b_0 was altered from the native protein value of −280 to values of around −175. This change was accompanied by a drop in the intrinsic viscosity from 0.36 to values ranging from 0.30 to 0.32. The ATPase activity of HMM was also sensitive to this detergent and, on the addition to the protein solution of SDS to a concentration of 1 mg/ml, the activity was immediately lost.

For the study of the effect of detergent on the non-water-soluble proteins, myosin and LMM Fr. I, Tris-HCl was used as a solvent, usually at a concentration of 0.5 M. Tris at this concentration had no effect on the exchange rates. In the presence of SDS the deuterium–hydrogen exchange of myosin was similar to that observed for HMM.

The most drastic effect of this detergent was noted for LMM Fr. I. At SDS concentrations of 10 mg/ml the exchange rate was extremely rapid and was complete within 15 min at pH 6.5 although a b_0 value of −290 was an indication that a considerable extent of helicity remained. However, at a lower detergent level of 1 mg/ml the infrared spectrum was complicated by the appearance of a new band at approximately 1590 cm^{-1} (Figure 5). At 5 mg/ml this band was prominent only after heating at 40°. For 10 mg/ml the band was not observed. This band was also occasionally found in the spectra of the other proteins but only after heating of the sample. As mentioned above this band could be similar to the 1608 cm^{-1} band found by Doty *et al.* (1958) for the β-configuration. Although it cannot be positively ascertained that the β-conformation was responsible for the 1590 cm^{-1} band, it is apparent that low concentrations of SDS caused some molecular

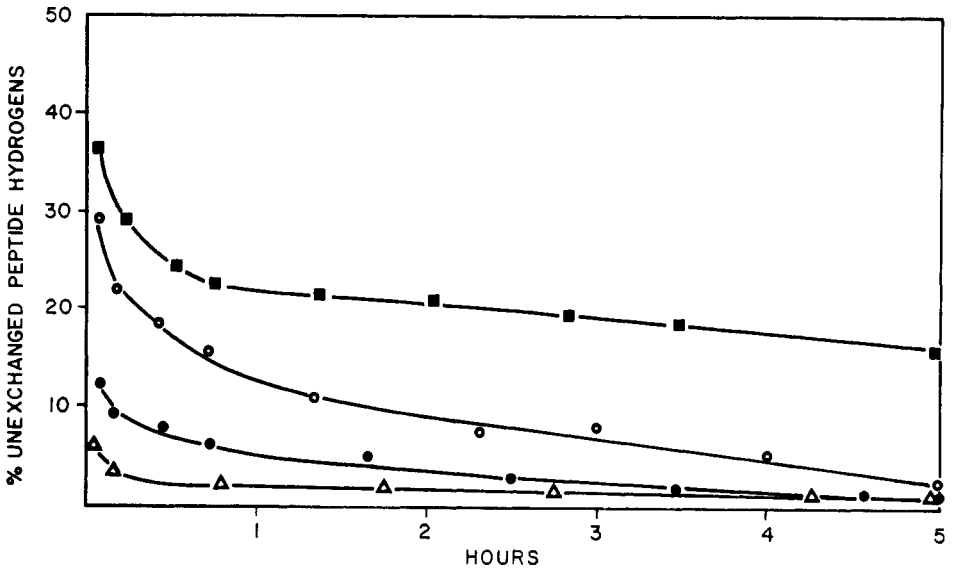

FIGURE 4: The effect of SDS on the deuterium-hydrogen exchange rates of the peptide hydrogens of HMM at SDS concentrations of 1 mg/ml (○), 5 mg/ml (●), and 10 mg/ml (△). (■), control sample.

rearrangement which was inhibited by higher concentrations of detergent.

SDS had less effect on the exchange of tropomyosin than on any of the other proteins. Although at concentrations of 10 mg/ml, SDS decreased the percentage of unexchanged hydrogens, exchange was not complete even after 5 hr at apparent pH values between 6.3 and 7.3.

Discussion

If it is assumed that the deuterium–hydrogen exchange of the random coil is relatively rapid (Blout *et al.*, 1961) and that in the pH range studied exchange would have been complete after 5 hr, then it is apparent that in all the native proteins studied there was some rate-limiting conformation. The similarity of the exchange of the highly helical proteins LMM Fr. I and tropomyosin is striking. In view of the rodlike character of these proteins it is unlikely that any extensive masking of the peptide hydrogens would occur, such as may be present in a globular protein. This being so, the exchange characteristics would represent the exchange of the peptide hydrogens involved in hydrogen bonding since both of these proteins have high helical contents.

Increased pH increased the exchange rate of these proteins but no structural changes were observed by optical rotatory dispersion measurements over the studied pH range. However, as Benson *et al.* (1964) point out, the possibility of more subtle conformational changes cannot be eliminated. Accepting Hvidt's (1964) theory on the mechanism of exchange, it can be stated that the time the peptide hydrogens spend in exchangeable form relative to the half-time of the exchange reaction increases with increasing pH. It is also reasonable to suppose that the peptide hydrogens of the random coil exist only in the exchangeable form. Hvidt further postulated that the exchange rate constants measure the free energy of the conformational transitions by which hydrogen atoms are exposed to the D_2O. If the free energy of these conformational transitions is related to conformational stability the exchange data would be a measure of the stability of a particular conformation. If this is so then the stability of the α-helix (tropomyosin, LMM Fr. I) shows a greater dependence on pH than does the exchange-limiting conformation present in HMM. Possibly the two conformations are influenced by different side chain interactions. Noelken and Holtzer (1964) found that tropomyosin was more stable to guanidine hydrochloride denaturation at pH 2 than at pH 7.4. They ascribed the stability at acid pH to interactions between side chain carboxyl groups.

Before it can be generalized, however, that the exchange rate of the α helix depends on the stability of that structure, further studies are necessary. Possibly structures of known high stability such as the copolymer of L-alanine and DL-glutamic acid, described by Gratzer and Doty (1963), could be used.

Whatever the ultimate significance of the exchange data may be, it is apparent that pH has a greater effect on the exchange of the α helix (tropomyosin, LMM Fr. I) than it has on the HMM exchange. This supports the conclusion of Benson *et al.* (1964) that deuterium–hydrogen exchange cannot be readily equated with helix content at arbitrarily selected pH values. Since the pH exchange dependence of HMM differed from that of the highly helical proteins it may be speculated that the exchange resistance of the HMM molecule was not due solely to the α helix but that some other conformation was also rate determining. Possibly as Leonard *et al.* (1965) suggested for albumin this region may result from masking by the tertiary structure. It is tempt-

ing to postulate that this region is part of the globular structure of the HMM molecule (Cohen, 1961) where the ATPase and actin combining capacity are thought to be located.

Wishnia and Saunders (1962), from an investigation of the exchange properties of guanidinated ribonuclease, suggest that the slowly exchanging hydrogens were those of the guanidinium groups. Such an explanation for HMM is unlikely on consideration of its amino acid composition compared with that of the other myofibrillar proteins (Kominz *et al.*, 1954).

For actin the smaller percentage of unexchanged peptide hydrogens at all pH values is indicative of less exchange rate-limiting conformations in this molecule compared with the other proteins studied. It is interesting that actin has the lowest reported helical content (Kay, 1958) of all the proteins studied here.

the available evidence the cause of the reduced helicity of the studied muscle proteins cannot then be attributed solely to altered hydrophobic interactions. The anionic nature of sodium dodecyl sulfate and possible electrostatic interactions with the protein side chains must also be considered. In the presence of 10 mg/ml SDS the exchange rates of LMM Fr. I and HMM were relatively rapid, although b_0 values indicated remaining helical contents of approximately 46 and 28%, respectively. The rate of deuterium–hydrogen exchange of the α helix then appears to vary considerably under different experimental conditions. It might also be expected that the exchange-rate determining effect of the α helix could vary in different proteins. Therefore as Leach and Hill (1963) point out, the number of hard-to-exchange hydrogen atoms in a given protein at a given pH has little significance with regard to any conformation without consideration of the effect of pH on its exchange rate. For proteins in which there is a possibility of more than one exchange rate-determining conformation (as in HMM) interpretation of the exchange data is more complicated.

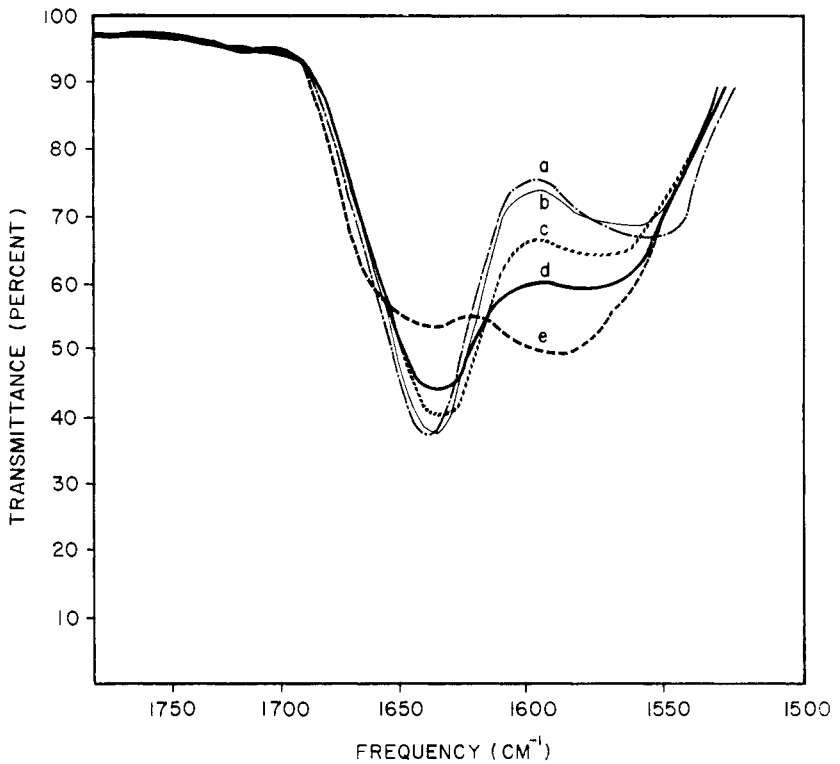

FIGURE 5: Infrared spectra of LMM Fr. I in the presence of 1 mg/ml SDS, apparent pH 6.45, recorded (a) 3 minutes, (b) 24 minutes, (c) 3.75 hr, (d) 8 hr, (e) 18 hr after the addition of D_2O to the lyophilized protein.

The action of sodium dodecyl sulfate on these proteins was to increase the exchange rate. Jirgensons (1962) and Meyer and Kauzmann (1962), working on reduced and carboxymethylated serum albumin and ovalbumin respectively, found that this detergent made the b_0 values more negative. Meyer and Kauzmann proposed that the detergent probably acted on hydrophobic bonds, thus enabling a higher helical content to form. The effect of ionic detergents on proteins depends both on ionic and adsorptive interactions. On

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