Tritium-Hydrogen Exchange in Keratins

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Received December 9, 1963

The rate of exchange of keratin fibers with tritiated water has been investigated under various conditions. There is a large equilibrium-isotope effect in the sense that tritium enrichment in the fiber phase appears to be greater than that in the solution phase by a factor of 1.28. There are four classes of exchangeable hydrogen atoms in keratin, distinguishable by their ease of exchange. In addition to rapidly exchanging hydrogen atoms, there are slower ones which show a *p*H dependence characteristic for structures which are stabilized by protonated side chains. A third class of hydrogen atoms exchanges only at elevated temperatures with a diffuse temperature dependence suggestive of structures such as α helices of various thermal stabilities. Before the fourth class of hydrogens can be exchanged, the keratin must be disrupted by drastic treatments with alkali or lithium bromide or certain reducing agents. The structures involved here are probably the stable multiple helices which are characteristic of many structural proteins. This interpretation of the rate data should permit use of the hydrogen-exchange technique for following the conformational changes in keratins and other structural proteins, which occur as a result of chemical treatment or mechanical deformation.

The structural proteins[1] are of biological necessity resistant to solvent action. Conformational studies of such proteins in solution are handicapped since methods of solubilization are sometimes so drastic that some of the structural features to be investigated are destroyed. Methods based on the rate of hydrogen exchange could play a unique role in investigating these systems, since they can be used equally well with proteins whether in the crystalline, fibrous, or solution states. In principle, hydrogen-exchange methods might therefore provide information about changes in conformation which occur in passing from one state to the other. However, this depends upon the successful interpretation of exchange data and the ability of the method to recognise structural features such as polypeptide chain helices.

In common with other proteins, keratins contain two main classes of exchangeable hydrogen atoms, namely, those which are replaced at a rate too rapid to measure, and those which exchange more slowly. While both classes of hydrogen atoms are located only at *N*,- and *O*,- sites in the protein molecule, the very reactive type has always been assigned to the more accessible and therefore "amorphous" regions of the keratin fiber, while the less reactive hydrogen atoms have been considered to lie in the less accessible and therefore "crystalline" regions.[2] In the past, several measurements have been made of the relative amounts of these two kinds of hydrogen atoms in order to determine the ratio of crystalline to amorphous material and its relationship to the fiber's physical and chemical properties.

The experimental methods used to follow hydrogen exchange have involved the use of 100% deuterated water. After the dried fiber has been exposed to deuterated water it is dried again, and the replacement of hydrogen by deuterium is estimated either gravimetrically (Burley *et al.*, 1955) or by infrared spectroscopy (Fraser and MacRae, 1958). More recently, tritiated water has been used in place of deuterated water for measuring hydrogen exchange in cellulose (Lang and Mason, 1960) and soluble proteins (Leach and Springell, 1962; Englander and von Hippel, 1962; Leach and Hill, 1963).

The present paper describes procedures for measuring tritium-hydrogen exchange in keratin fibers exposed to tritiated water. The objective is the interpretation of exchange data in terms of the various structures present in fibrous keratins, so that the hydrogen-exchange technique may be utilized in conformational studies on these and other structural proteins.

MATERIALS AND METHODS

Keratins.—Lincoln wools[3] (SW 14, MW 114, and MW 144), Merino wools (fabric MW 27 and wool top MW 118), and horse-tail hair were washed with petroleum ether, chloroform-methanol (1:2), followed by ethanol and water. One sample was then washed with a nonionic detergent in acetate buffer (*p*H 5, 40°) followed by water.

Chemical Treatments and Methods.—The buffers used were potassium tetroxalate (*p*H 1.7), potassium phthalate (*p*H 3.1), sodium acetate (*p*H 5.0), potassium phosphate (*p*H 6.9 and 12.3), and sodium borate (*p*H 9.0 and 10.0), and the concentration of these ions during the exchange experiments was 0.025 M or less.

Disulfide-bond fission to produce "zero-disulfide" wool was carried out at *p*H 9 using sodium sulfite and excess methylmercuric iodide as described elsewhere (Leach, 1960; Maclaren *et al.*, 1960).

Treatments with lithium bromide to produce contraction of wool were carried out either by (a) heating the wool samples with lithium bromide (8 M, 100°, 18 hours) in the *presence* of tritiated water, or (b) pretreating the wool with lithium bromide (6 M, 100°, 1.5 hours) in potassium succinate buffer (0.1 M, *p*H 6) *before* commencing the tritium-hydrogen exchange experiment. In the first case there were 7% weight losses with Lincoln wools and 10% weight losses with the Merino fabric.

The estimation of amino-N made use of the colorimetric ninhydrin reaction, with pyridine to extract the color from the wool (see, e.g., Leach and Hill, 1963). Values are quoted in the text in terms of "leucine

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[1] The structural proteins are here taken to include the keratin, myosin, elastin, and fibrinogen group.

[2] The identification of the inaccessible and accessible with the "crystalline" and "amorphous" regions leads to difficulties which are discussed at the end of this paper.

[3] Sample numbers are those used in previous investigations in these laboratories. Unless otherwise stated the sample most frequently used was Lincoln MW 144.

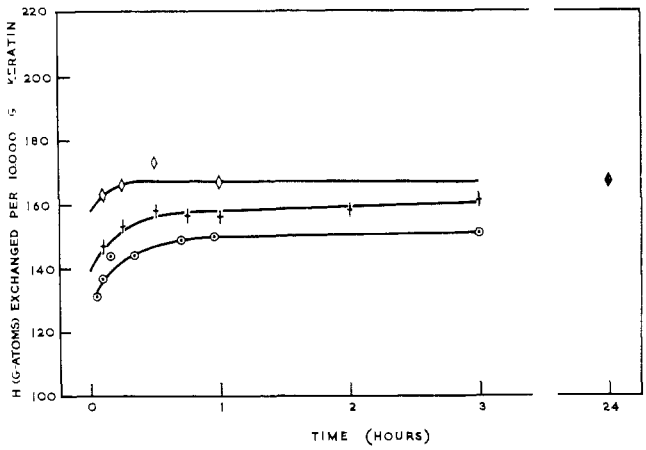

FIG. 1.—Rates of exchange of wool fibers with tritiated water at 20°. +, three Lincoln wools providing an identical rate curve, whether in the presence or absence of nonionic detergent at *p*H 5; ◊, rate curve at *p*H 5 for one of these wools (MW 114) after pretreatment with potassium carbonate (see text); ⊙, rate curve for wool MW 114 at *p*H 3.1. The ordinates in all figures are uncorrected for the equilibrium-isotope effect mentioned in the Discussion.

equivalents" since leucine is used as a color standard; values include the color due to the 8 side-chain amino groups of lysine residues. The solubility of wool in solutions of bisulfite and urea was determined by the method of Lees and Elsworth (1955).

Tritiated water (5 c/ml) from the Radiochemical Center, Amersham, England, was diluted to 2 mc/ml with deionized water. Radioassays were carried out on 10–100 mμc of tritium per aliquot using a Packard Tri-Carb liquid scintillation spectrometer (Series 314 EX) with the settings and scintillation mixture described by Leach and Hill (1963). Within the experimental range of activities, the count-rate was linear with respect to the amount of tritium.

Exchange Procedure.—Wool samples (20 mg, 3 replicates) were weighed and equilibrated at 25° overnight with buffer solutions (0.05 M, 1 ml) in reaction flasks (10 ml, B14 cones). In some cases BRIJ nonionic detergent (1 ml/liter) was present to ensure wetting. To commence the exchange experiment, tritiated water (2 mc/ml, 1 ml) was added at 25° and left for periods of 45 seconds up to several days. The excess solution was then removed by pipet, and the remaining fibers were frozen at −60° and freeze-dried *in vacuo* using the manifolds and vacuum system described elsewhere (Leach and Springell, 1962; Leach and Hill, 1963). Drying was carried out for 1.5 hours at room temperature, then for 1 hour at 40°, and finally for 2 hours at 70°. The fibers were then dissolved in KOH (0.2 M, 2 ml) by warming for 1–2 hours at 90–95°.

Radioactivity was estimated on an aliquot (50 μl, containing *ca.* 0.5 mg wool) which was added to water (1.95 ml) and scintillation mixture (15 ml). The presence of KOH made it unnecessary to add tetraethylammonium hydroxide (Leach and Hill, 1963) in order to keep the partially hydrolyzed protein in solution. The count was compared with standards made up from the original reaction mixture by diluting 100 μl to 50 ml with water and taking 50 μl for radioactive assay.

Since a single molecular weight cannot be quoted for a keratin, the extent of exchange is expressed throughout as the number of g-atoms of hydrogen exchanged per 10,000 g of dry keratin, and this was calculated from the expression:

$$\frac{4.444 \times 10^3}{wP} \frac{(S_t - B)}{(S_o - B)}$$

where the numerical coefficient takes into account the amount of hydrogen in a 50-μl aliquot of the original reaction mixture and also corrects for the fact that only a fraction of the original wool is counted. S_t is the count (cpm) for the sample (50 μl) taken at time t; S_o is the count (cpm) for the standard, diluted as above; B is the count (cpm) for a blank vial containing all ingredients except tritium; w is the amount of wool originally weighed out (mg); and P is the fraction of the weighed sample which is dry keratin.

RESULTS[4]

Rates of Exchange at 20°.—Lincoln wools of different origin were indistinguishable in their rates of exchange with tritiated water at *p*H 5 and 20°. The results are shown in Figure 1 (middle curve). In each case there was a rapid initial exchange so that by the time the first measurement was taken (in this case 3 minutes), the number of g-atoms of hydrogen which had exchanged was 145–150/10,000 g. Exchange then proceeded more slowly until the value reached 167 g-atoms after 24 hours. Beyond this point, exchange continued too slowly for any increase to be detectable over a period of 7 days.

The presence or absence of BRIJ nonionic detergent made no detectable difference to the measured rate. This is seen more clearly using horsehair, where the rates of exchange were considerably slower than for wool during the first 20 minutes. Figure 2 shows that even during the earliest stages of the exchange detergent had no effect on the rate.

Wools which had previously been dried by heating for 3 hours at 105° showed no change in their rates of exchange. However, pretreatment of wool with potassium carbonate (0.05 M, 55°, 3–4 hours), which decreased its urea-bisulfite solubility from 31 to 4%, had a marked effect upon its rate of exchange with tritiated water (Fig. 1, upper curve). The maximum exchange of 167 g-atoms of H at 20° occurred within 30 minutes and there was then no further increase even after 24 hours.

Effect of Temperature on Maximum Exchange.—The maximum hydrogen exchange occurring after 24 hours at *p*H 5 and 20° was 167–169 g-atoms not only for various Lincoln wools but also for two samples of Merino wool. In each case, however, this value could be exceeded by tritiating at higher temperatures. In Figure 3 the total exchange occurring after 24 hours at temperatures between 25 and 100° is shown. It may be seen that the total hydrogen exchange gradually increased to a maximum of about 190 g-atoms at 100°.

Effect of pH on Exchange.—The number of g-atoms of hydrogen exchanging instantaneously at 20° increased with increasing *p*H from 135 at *p*H 1.7 to 204 at *p*H 12.3. This is shown in Figure 4 (lower curve). The *p*H dependence is not simple: In the *p*H ranges 1–3 and 5–7 there is little effect of *p*H on the rate of exchange, whereas there are marked effects of *p*H in the ranges 3–5 and 8–12, where ionization of protein side chains is known to occur. Specific ion effects are discounted because experiments at *p*H 5 showed no effect on the rates of exchange when the ionic strength was increased up to 4-fold.

[4] Since 100% tritiated water is never used, it should be understood that whenever the text refers to the "number of g-atoms of tritium" the tritium is in fact considerably diluted with hydrogen.

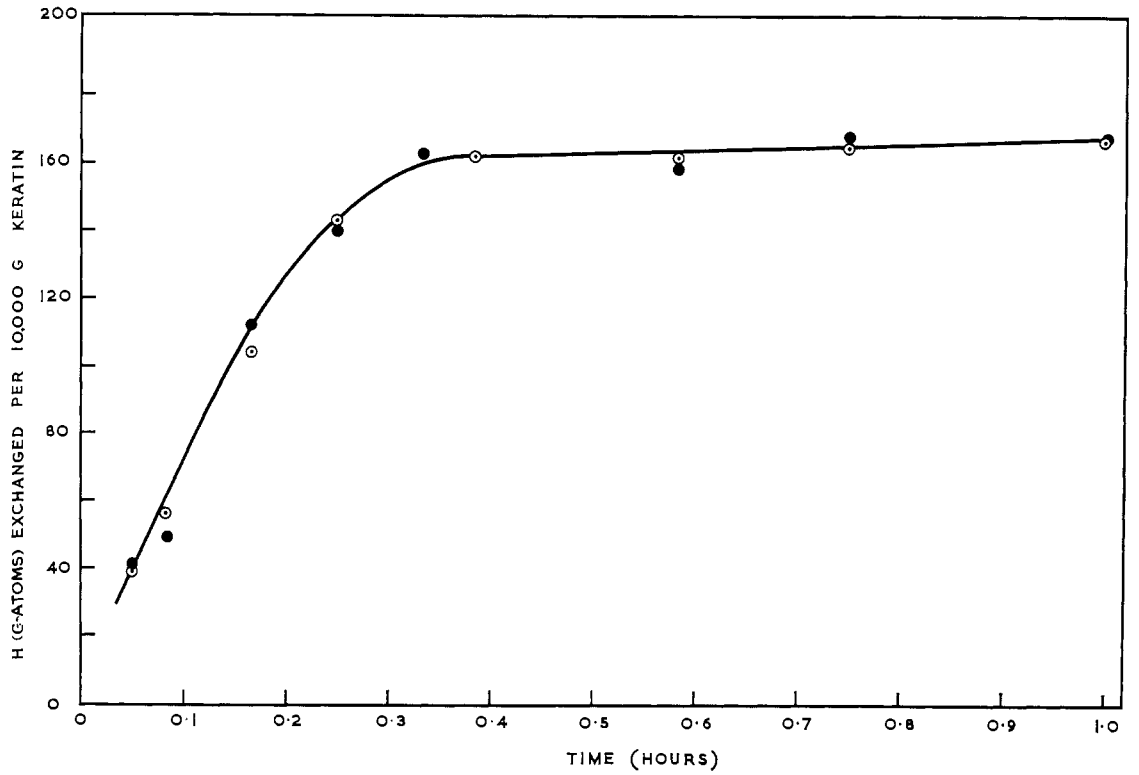

FIG. 2.—The rate of exchange of horsehair with tritiated water at *p*H 5 and 20°. ●, in the absence of detergent; ⊙, in the presence of nonionic detergent.

The number of g-atoms of hydrogen exchanging after 24 hours at 20° is shown in the upper curve of Figure 4. There is little effect of *p*H between 1 and 9 where the exchange amounts to 160–167 g-atoms. Above *p*H 9, however, there is a sudden increase from 166 to 220 g-atoms of hydrogen exchanging at *p*H 12.3. This is the *p*H region in which there is a build-up of negative charges along the protein chains, and keratin fibers are subject to alkaline swelling.

Effect of Prolonged Treatments of Wool with Tritiated Water.—Exchange measurements at 20° were carried out for periods up to 9 days. As already pointed out, the value of 160–167 g-atoms was reached after a few hours and increased only slowly beyond this point. After 3 days the mean value reached for all *p*H values between 2 and 9 was 182 ± 5 and there was no significant increase up to 9 days. However, a difference was noticed in the ease with which the incorporated tritium could be removed from the wool when the labeling had been carried out for different lengths of time. The washing conditions used, viz., 18 hours at *p*H 7 and 20°, were capable of removing all but 2.5% of the tritium which had been incorporated by exchanging for, say, 3 days at *p*H values between 2 and 7. Prolonged tritiation at 20°, while leading to no increase in the total uptake, allowed more of the tritium to become "trapped," so that after 9 days as much as 8% of the incorporated radioactivity could no longer be removed by the standard washing conditions. At *p*H 9 this amount of tritium was "trapped" more rapidly than at lower *p*H values.

At *p*H 12 the total tritium uptake reached a maximum of 210–220 g-atoms of hydrogen of which 12% could not be washed out after only 3 days tritiation. The amino-N groups of wool, measured over a period of 7 days at *p*H 12.2 and 20°, unexpectedly showed no increase in the value of 1.61 leucine equivalents per 10,000 g, showing that none of the extra exchange

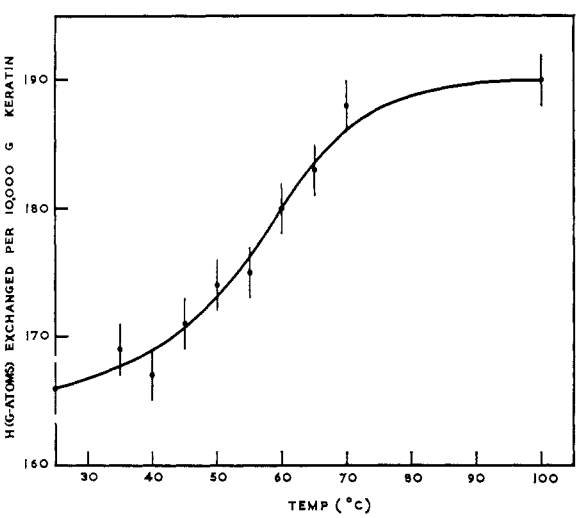

FIG. 3.—The effect of temperature on the final extent of tritium-hydrogen exchange in wool after 24 hours with tritiated water.

was due to the appearance of amino and carboxyl groups caused by peptide-bond fission.

Effect of Lithium Bromide Treatment and Disulfide-Bond Fission.—Concentrated lithium bromide solutions have a disordering effect on proteins. The visible effect on keratin fibers is to cause a contraction in length and in this state the X-ray diffraction pattern is destroyed. Pretreatment of wool with lithium bromide (6 M, *p*H 6, 100° for 1.5 hours) resulted in a greatly increased tritium exchange at 20° and *p*H 5, namely, 203 g-atoms of hydrogen instead of the usual 167. All but 0.5% of this incorporated tritium could be removed by washing at 20°.

Tritiation at 100° of the lithium bromide–treated wool resulted in no greater exchange (193 g-atoms)

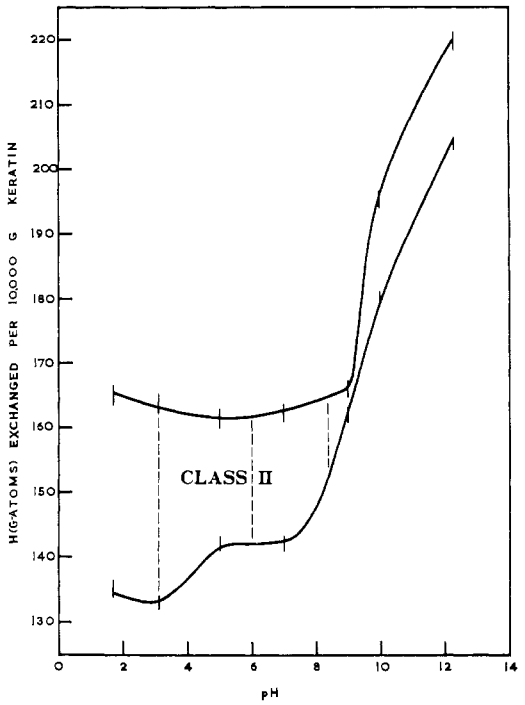

FIG. 4.—The extent of exchange of wool with tritiated water buffered at various *p*H values at 20°. Lower curve, after 3–5 minutes; upper curve, after 24 hours. The vertical distances (broken lines) correspond to the number of g-atoms of hydrogen exchanging slowly during a 24-hour experiment, i.e., hydrogen atoms of class II.

than at 20°, but under these conditions a larger proportion of tritium atoms were "trapped" (14%) and could no longer be washed out at 20°. The results are tabulated in Table I, which shows that the effects of pretreatment with lithium bromide on horsehair were very similar to those on wool.

When wool was treated with lithium bromide (8 M, 100°, 18 hours) *in the presence* of tritiated water, the total exchange increased to 211 g-atoms, a value which appears to be the upper limit for exchange under any of the conditions used.

Table I shows that when lithium bromide–treated wool was heated at 100° for 20 hours at *p*H 5 the effect of the lithium bromide was partly nullified. That is, in subsequent tritium-hydrogen exchange at 20° and 100° the wool exchanged slowly once again and behaved almost as though it had received no preliminary treatments at all.

The effect of complete fission of all disulfide bonds is seen in Table I. At 20° the exchange reached 188 g-atoms of hydrogen after 24 hours and this increased very little when the exchange was carried out at 100°. However, after tritiation at the higher temperature, 10% of the incorporated tritium could no longer be washed out using the standard conditions, whereas the corresponding figure after tritiation at 20° was less than 1%.

TABLE I

EXCHANGE OF KERATINS[a] WITH TRITIATED WATER

Pretreatment	Temp of Exchange (at *p*H 5 for 24 hr) (°C)	Extent of Exchange (g-atoms of hydrogen or tritium/10,000 g dry wool)	Gram-Atoms of Tritium Remaining after Washing (at *p*H 5 for 16 hr at 20°)
None	20	167 (164)	4
	100	186 (184)	30
LiBr	20	203[b]	<1
	100	193	26
LiBr (horsehair)	20	197	
	100	197	
LiBr during tritiation	100	211 (207)[b]	
LiBr, then specimen heated at *p*H 5, 100° for 20 hr before tritiation	20	178	
	100	186	
Disulfide-bond fission	20	188	8
	100	193	20

[a] Lincoln wool MW 114 (solvent-scoured and deionized) except where otherwise stated. The figures in parenthesis are mean values of four different wools of varying fiber diameter and origin. Figures are uncorrected for the equilibrium-isotope effect mentioned in the Discussion. [b] Tests for C-tritiation were negative (see text).

DISCUSSION

Maximum Hydrogen Exchange.—Keratins do not differ widely in composition and the amino acid analyses of the various keratins used in the present work lead to a calculated value of 160–165 g-atoms of hydrogen for the total exchange at *p*H 6–7. This is of course based upon the supposition that all hydrogen atoms attached to nitrogen, oxygen, or sulfur atoms in peptide bonds and side chains can be replaced, but not those attached to carbon atoms. The total number of g-atoms of hydrogen exchanging under the most rigorous conditions, viz., 211/10,000 g. of keratin,[5] is greatly in excess of the calculated value. Additional exchange at carbon atoms would explain the high values. However, it has been well established in exchange work with globular and fibrous proteins that exchange at carbon atom sites occurs only in strong alkali or hot acid (J. Hill and S. J. Leach, in preparation). Concentrated lithium bromide seems unlikely to cause exchange at carbon sites since we have seen that practically the whole of the tritium incorporated at 20° can be readily removed by exchange with water at 20° and neutral *p*H. Tritiation of ribonuclease at 100° in the presence of lithium bromide confirmed this opinion, since dialysis against water at *p*H 5 removed all but 0.2 g-atom of tritium per mole of ribonuclease.

Several previous workers have reported tritium-hydrogen exchange values in excess of those calculated from the known chemical structure. As in the present investigation, all were concerned with heterogeneous systems. Lobunez and Karush (1959) noted that, in partitioning tritium between solid protein and gaseous ammonia, the heavy isotope favored the protein. In vapor-liquid partition of tritium with tritiated water, the condensed phase had the higher specific activity (Sepall and Mason, 1960). A closer parallel to the present work is to be found in that of Lang and Mason (1960), who observed that when cellulose was equilibrated with tritiated water vapor the heavy isotope "favored" the fiber. The magnitude of this isotope effect was given by α, where

$$\alpha = \frac{\text{T/H ratio in solid phase}}{\text{T/H ratio in water}}$$

[5] The slightly higher value observed at *p*H values above 12 is probably due to a small amount of tritium exchange at α-carbon sites (J. Hill and S. J. Leach, in preparation).

The α was 1.29 and very similar to the value observed here for the ratio of observed to calculated maximum exchange.

It seems reasonable to assume that the discrepancy observed with keratin is due to the existence of an equilibrium-isotope effect. Since cellulose and keratin differ so markedly in chemical structure, the similarity in value of α suggests that the distribution of the isotope is determined primarily by the nature of the phase heterogeneity rather than the nature of the chemical sites at which the hydrogen atoms are bound. The possibility that α may vary from site to site cannot be dismissed and should be investigated using, say, insoluble polyamino acids containing only one kind of site. Meanwhile a value of 211 may be taken as an empirical maximum value for the number of g-atoms of exchangeable hydrogen per 10,000 g of keratin. Smaller amounts of exchange will be expressed as a proportion of this value. Using this procedure, the extent of exchange at 20° after 48–72 hours is 80% of the maximum value. This agrees quite well with the value of 83% found by Burley *et al.* (1955) for wool using 100% D_2O, where no isotope effect would be observable.

Interpretation of Slow Exchange.—The experimental results are summarized diagrammatically in Figure 5 where the exchangeable hydrogen atoms in wool keratin are divided into four classes according to their ease of exchange with tritiated water. Before the relationship of each class to molecular structures in keratin can be traced, the possible reasons for slow hydrogen exchange must first be discussed.

One possible reason for slow exchange is limited accessibility at the *histological* level, e.g., diffusion across membranes and through structures of varying pore size. These factors, and the related one of "wetting," may be ignored in the present work for several reasons. Rates of water sorption (I. Watt, private communication) show that "sorption equilibrium" between keratin fibers and water is complete before the first exchange reading is taken. Second, rates of exchange were unaffected by detergents (Figs. 1 and 2). Finally, all six types of keratin, of widely different fiber diameter, show closely similar exchange characteristics (see Table I). Insofar as hydrogen exchange is a reflection of molecular order, it is apparent that the general organization of these keratins is probably similar.

Slow exchange at the *molecular* level may arise as a result of either limited accessibility or low reactivity—a distinction which is not always recognized. Until a few years ago, slow exchange in globular proteins was invariably attributed to low reactivity caused by hydrogen bonding; slow exchange in keratins, on the other hand, was ascribed to inaccessibility and indeed formed the basis of "accessibility" measurements. More recently protein chemists have invoked both factors to explain the physicochemical properties of globular proteins. They recognize the existence not only of hydrogen-bonded structures but of impenetrable, probably apolar regions which may contain either helical or random chains or both, and which will not react or exchange until the whole region is opened up and exposed to the aqueous medium. Parallel with this development, work on the various structural proteins has revealed the presence of highly organized structures such as multiple helices and lamellae of extended chains, either of which might well offer steric interference to the approach of water molecules and thereby inhibit exchange.

Unfortunately there has not yet been any systematic work on the hydrogen-exchange kinetics of polyamino

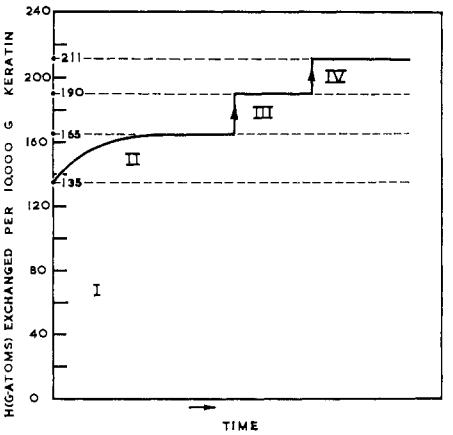

FIG. 5.—Diagrammatic summary of tritium-exchange results showing the four classes of hydrogen atoms in wool keratin. I, rapid; II, slow but *p*H dependent; III, thermally labile; IV, most refractory.

acids or other model systems of well-defined conformation, so that there is no unequivocal basis for interpreting the exchange data obtained from proteins. However, an interpretation will be attempted on the basis of the conformational features discussed above.

Class I.—By analogy with work on deuterium-hydrogen exchange with small peptides and denatured proteins, the hydrogen atoms of class I, that is, those exchanging too rapidly to measure, must be located at readily accessible sites and are not involved in inter- or intramolecular hydrogen bonding (see, e.g., reviews by Leach, 1959; Scheraga, 1961). The molecular regions concerned are therefore disorganized and might be located in the so-called "amorphous" regions. They comprise 64% of all exchangeable hydrogen atoms.

Class II.—Class II hydrogens, comprising 14% of all exchangeable hydrogens at acid *p*H, exchange sufficiently slowly for their rates to be measurable over a few hours at room temperature. The number of hydrogen atoms in this class depends upon the *p*H at which the measurements are made, and is given by the vertical distance between the upper and lower curves of Figure 4. The lower curve shows the amount of exchange after 3–5 minutes while the upper curve shows the exchange after 24 hours. In acid solution, we might have expected that the increased positive charge and resultant electrostatic repulsions between (and along) polypeptide chains would favor unfolding and therefore more rapid exchange. Instead we find that the number of slowly exchanging atoms of class II is maximal in acid solution and diminishes at higher *p*H values until it is zero at *p*H 9. That is, at *p*H 9 the number of g-atoms of hydrogen exchanging after 3–5 minutes is almost the same as that exchanging after 24 hours, namely, 165.

Between *p*H 1.5 and 9 the lower curve of Figure 4 resembles a protein titration curve and this suggests that the hydrogen atoms of class II are located in regions which change in reactivity according to the state of ionization of side chains. Of the 32 g-atoms of hydrogen in class II at *p*H 1.5–3, only 9 could possibly be accounted for by protonation of carboxyl side chains. However, interactions between such protonated carboxyl groups could well lead to slow exchange at some of the —CONH— sites in the polypeptide chains. This type of main-chain stabilization is believed to occur in acid solutions of bovine serum albumin (Loeb and Scheraga, 1956), ribonuclease (Leach and Hill, 1963), poly-L-glutamic acid (Doty

et al., 1957), and copolymers of L-lysine and L-glutamic acid (Doty and Imahori, 1957; Doty *et al.*, 1958). The copolymer example illustrates the high charge densities which can be supported by folded segments of polypeptide chains when they are stabilized by interactions between carboxyl side chains.

The hydrogen atoms of class II depend not only upon alkali-labile interactions for their slow exchange but also upon disulfide links. When the stabilizing disulfide crosslinks are removed, even using highly specific methods of disulfide fission, the hydrogen atoms of this class no longer exchange slowly even in acid solution (see Table I).

Class III.—The structures in which the less reactive hydrogen atoms are located can be partly disrupted at elevated temperatures even at neutral *p*H. The number of g-atoms of hydrogen exchanging after 24 hours at *p*H 5 increases from 165 at 20° to a value approaching 190 at 100° (Table I). The form of the temperature-dependence curve (Fig. 3) is sigmoid, showing a gradual increase in exchange with increase in temperature around a mid-point of about 55°. Reversible curves of this type are obtained with various polyamino acids and proteins when conformation-dependent physical properties are plotted as a function of temperature. They are often indicative of transitions from ordered to disordered states (see, e.g., reviews by Leach, 1959, or Scheraga, 1961). The failure of hydrogen atoms of class III to exchange without heating and their characteristic temperature-dependence curve suggest by analogy that they are involved in peptide hydrogen bonds located in α helices. It is well known that α-keratins show X-ray spacings characteristic of the α helix though the meridional arc at 5.1 A suggests that some or all of these must be distorted (see, e.g., Fraser and MacRae, 1961). The broad temperature dependence shown by Figure 3 suggests that the helical regions are not all of equal stability, differing perhaps in length and/or the extent to which they are stabilized by side-chain interactions and disulfide links. Stabilization by disulfide links must be of great importance since, in common with the less stable hydrogens of class II, the whole of class III becomes exchangeable even at room temperature when the disulfide links are broken. Once these links are removed, heating even at 100° has little or no further effect on the exchange. The extra tritium atoms exchanged at 100° cannot be removed readily at lower temperatures: washing at 20° leaves 30 g-atoms of tritium in the keratin. However, substantially all of the tritium incorporated at 100° can be removed by washing at 100°. This means that the helices are reversibly opened up at 100°, refold at 20° to trap the extra hydrogen, and can be unfolded once again at 100°. Reversibility of thermal unfolding is indicated also by the fact that heating dry wool for 3 hours at 100° has no effect on subsequent exchange rates measured at 20°.

Class IV.—Heating alone is insufficient to achieve complete exchange in keratins. To exchange the hydrogen atoms of class IV, i.e., those between 190 and 211 in Figure 4, the keratin structure must be completely disrupted either by titration to *p*H 12, by treatment with concentrated lithium bromide, or by complete disulfide-bond fission, using thiols and alkylating agents[6] rather than the sulfite-mercurial method described in the experimental part. It is significant that the conditions required to achieve complete exchange of class IV hydrogen atoms are those which lead to destruction of crystalline order as seen in X-ray diffraction patterns, and can ultimately lead to dissolution of the fiber. Hydrogen atoms as refractory as this are rarely to be found among the globular proteins, although it has been reported that β-lactoglobulin exchanges incompletely even on prolonged heating (Linderstrøm-Lang, 1955). The necessity for such destructive pretreatments suggests that the structural features responsible for this refractory behavior lead to inaccessibility rather than lowered reactivity. It seems most likely that the hydrogen atoms of class IV are peptide hydrogen atoms located in stable multiple helices of the type suggested by Crick (1952), Pauling and Corey (1953), Skertchly and Woods (1960), Lang (1956), or Fraser and MacRae (1961). Hydrogen exchange becomes possible only when the helical strands have been separated and unfolded by treatment with lithium bromide or alkali. The difference in stability between the structures of classes III and IV would then be the difference between single and multiple helices. Parallel situations are to be found among the myosins.

Effect of Heat on Reduced or LiBr-treated Keratin.—Wools which have been rendered completely accessible by reduction or lithium bromide treatment are unstable to heat, which appears to close up the structure again. Thus (1) when lithium bromide–treated wool was heated at 100°, subsequent exchange either at 20 or 100° was incomplete (178 and 186, respectively, see Table I), and (2) tritiation at 100° in the presence of lithium bromide was partly irreversible: 20–30 g-atoms of tritium were "trapped" in the structure and could not be removed by washing. The effect of heat on reduced and alkylated keratins produced similar "collapsed" structures which were incompletely penetrable by tritium atoms trapped inside, or by tritiated water outside. X-ray diffraction patterns of the heated keratins showed that the original α pattern had not returned but a small amount of oriented β material was now present. The effect of heat on the disrupted multiple helices is probably to produce a tangle of disordered plus partly orientated β chains containing impenetrable regions.

Effect of High pH on Keratin.—Above *p*H 9 the number of g-atoms of hydrogen exchanging at 20° increases rapidly beyond 165, i.e., beyond the limits of class II (see Fig. 4), until at *p*H 12 all hydrogen atoms exchange and the value reaches 220.[7] The marked increase in rates of exchange above *p*H 9 runs parallel with a general increase in chemical reactivity of keratins in this *p*H range. For example, extensive disulfide-bond reduction is easier to achieve at *p*H values above 9. The enhanced sensitivity of wool to structural changes in mildly alkaline solution is illustrated by the effect of brief pretreatments with warm potassium carbonate. Figure 1 shows that the previously slowly exchanging hydrogen atoms of class II will then exchange within a few minutes even at *p*H 5. It appears that irreversible structural changes occur very readily at *p*H 10 even at 40°. At this *p*H the disorganizing influence of charge repulsions along the chains is no longer offset by carboxyl-carboxyl interactions. The disorganization which occurs must alter the location of the carboxyl groups relative to each other, so that when the wool is titrated back to *p*H 5 the side-chain interactions can no longer re-form and exchange occurs more rapidly than is normal at this *p*H

The conformational changes occurring at *p*H 10 and 40° are no doubt the main reason for the marked de-

[6] A comparative study of the effect of different procedures for breaking disulfide bonds is in progress.

[7] This value and all others quoted in the Discussion are uncorrected for the equilibrium-isotope effect described at the beginning of the Discussion.

crease in the solubility of keratins in urea-bisulfite or thioglycollate solutions after such treatments. These changes in solubility have been attributed variously to disulfide rearrangement (Kessler and Zahn, 1958) and formation of lanthionine crosslinking (Lees and Elsworth, 1955). Whether or not such changes occur concomitantly, the exchange results support the suggestions of Speakman (1959) and Swan (1959) that the main cause of the decreased solubility lies in major conformational changes in the keratin.

A parallel for such conformational changes might be seen in the α-β transformation which occurs on heating poly-L-lysine at *p*H 10.8 (Applequist and Doty, 1962). A transformation of this kind occurs when keratin fibers are stretched and this change can be made permanent by steaming in the β state. In this state the urea-bisulfite solubility is reduced (Lees *et al.*, 1960). If β material were formed in keratin by heating in alkali, it would most probably be disorientated, and unlike the highly organized β structures of, say, feather keratin or silk fibroin, would be rapidly exchangeable.

Use of Exchange Data.—Two assumptions have frequently been made about hydrogen-exchange rates in keratins, namely, that *all* slow hydrogen atoms are located in the peptide bonds rather than the side chains, and that the ratio of slow hydrogens to fast ones can provide an estimate of the proportion of crystalline material in the keratin. Such assumptions are difficult to justify on the basis of exchange work on simpler systems (e.g., Harp and Eiffert, 1960; Leach and Scheraga, 1958). The large number of slow hydrogen atoms and the nature of their temperature- and *p*H dependence make it unlikely that either assumption is applicable to keratins. The difficulties inherent in making estimates of crystallinity and the wide variations in these estimates have been reviewed by Fraser (1955), Fraser and MacRae (1958), and Peters (1963). Estimates vary from 10 to 60% depending on the method of measurement and the assumptions made in interpreting the estimates. In any event, the usefulness of these estimates is doubtful since the term "crystallinity" is open to several definitions. For example, insulin exchanges more readily with D_2O even in the insoluble, isoelectric, and highly crystalline state than does fibrous keratin which is considerably less "crystalline."

The present work suggests that 30 g-atoms of hydrogen (class II) are located both in side chains and in peptide bonds, 25 (class III) are probably in the peptide bonds of single helices, and 21 (class IV) are most likely the peptide-bond H atoms of multiple helices. An estimate of the proportion of polypeptide chains in the most stable, probably multiple-helical, form would be 21/105 or 20%. If the less stable hydrogen atoms of class III were included, the proportion of chains in both forms would be 46/105 or 44%. These estimates need bear no simple relationship to "crystallinity," but should be useful in comparative studies aimed at understanding the conformational changes which accompany physical or chemical modifications of keratins. A point which arises from the *p*H dependence shown in Figure 4 is that comparisons of keratin-exchange data are best made in acid solutions where rates are slowest and differences are likely to be greatest.

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CORRECTION

In the paper by M. Rosell-Perez and J. Larner, in Volume 3, No. 1, January, 1964, on p. 86, column 2, lines 22-23 " . . . converted to an *I* form (Fig. 7), . . ." *should read,* " . . . converted to an *I* form (Fig. 4), . . ." On pp. 87 and 88 the legends for Figs. 6 and 7 have been transposed; i.e., Fig. 6 appears on p. 87 with the legend to Fig. 7, and Fig. 7 appears on p. 88 with the legend to Fig. 6.

Through an error, the last article in the April, 1964 issue was omitted from the Table of Contents and the author's names were not included in the Author Index. The article, "Synthesis of *N*-Monomethyl- and *N*,*N*-Dimethylcephalins," by David Shapiro and Yechiel Rabinsohn, appears on page 603 of Volume 3, No. 4.