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Treatment methods for the determination of δ^2H and δ^{18}O of hair keratin by continuous-flow isotope-ratio mass spectrometry

Gabriel J. Bowen[1]*, Lesley Chesson[1], Kristine Nielson[1], Thure E. Cerling[1,2] and James R. Ehleringer[1,3]

[1]Department of Biology, University of Utah, Salt Lake City, UT 84112, USA
[2]Department of Geological Sciences, University of Utah, Salt Lake City, UT 84112, USA
[3]IsoForensics Inc., 423 Wakara Way, Salt Lake City, UT 84108, USA

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The structural proteins that comprise ~90% of animal hair have the potential to record environmentally and physiologically determined variation in δ^2H and δ^{18}O values of body water. Broad, systematic, geospatial variation in stable hydrogen and oxygen isotopes of environmental water and the capacity for rapid, precise measurement via methods such as high-temperature conversion elemental analyzer/isotope ratio mass spectrometry (TC/EA-IRMS) make these isotope systems particularly well suited for applications requiring the geolocation of hair samples. In order for such applications to be successful, however, methods must exist for the accurate determination of hair δ^2H and δ^{18}O values reflecting the primary products of biosynthesis. Here, we present the results of experiments designed to examine two potential inaccuracies affecting δ^2H and δ^{18}O measurements of hair: the contribution of non-biologic hydrogen and oxygen to samples in the form of sorbed molecular water, and the exchange of hydroxyl-bound hydrogen between hair keratin and ambient water vapor. We show that rapid sorption of molecular water from the atmosphere can have a substantial effect on measured δ^2H and δ^{18}O values of hair (comprising ~7.7% of the measured isotopic signal for H and up to ~10.6% for O), but that this contribution can be effectively removed through vacuum-drying of samples for 6 days. Hydrogen exchange between hair keratin and ambient vapor is also rapid (reaching equilibrium within 3–4 days), with 9–16% of the total hydrogen available for exchange at room temperature. Based on the results of these experiments, we outline a recommended sample treatment procedure for routine measurement of δ^2H and δ^{18}O in mammal hair.

Mammal hair is composed almost entirely of α-keratin, a structural protein. Because hair keratin is formed continuously during hair growth and is resistant to chemical and biological alteration following its formation,[1,2] stable isotope ratios of hair samples may preserve temporally resolved information about the location and/or physiology of animals throughout the period of hair growth.[3–5] Several recent studies have investigated the relationship between the stable isotope ratios of mammal hair and their environment. Most of this work has focused on carbon and nitrogen isotopes, which have demonstrated capacity to provide information on dietary composition and nutritional status, both in terms of average values[2,6–8] and time series.[4,5,9] In contrast, the utility of hydrogen and oxygen isotope analysis of keratin has been demonstrated in ecological and forensics studies of invertebrates and birds,[10–12] but only one study has reported H- and O-isotope values for mammal hair.[13]

The stable isotopes of hydrogen and oxygen are ideally suited to applications involving reconstruction of the origin or movements of animals. Hydrogen and oxygen, as the elemental constituents of water, are distributed within the hydrologic cycle in a systematic way.[14] In many regions, the resulting geospatial patterns in the water isotope composition of terrestrial environmental waters are relatively simple and robust, and provide a basis for interpreting the origin of animal tissue samples that incorporate the environmental water signal.[15] In order to allow the successful and widespread application of this method in the ecological and forensic sciences, however, standard and well-tested methods for the measurement of H- and O-isotope ratios in organic substrates are required. In this study, we explore and refine a method for the treatment of mammal hair samples prior to H- and O-isotope analysis by high-temperature conversion elemental analyzer/isotope-ratio mass spectrometry (TC-EA/IRMS). Our experiments are designed to test the importance of potential sources of contamination in

**Correspondence to*: G. J. Bowen, Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112, USA.
E-mail: gbowen@biology.utah.edu
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measurements of the isotopic composition of hair: (1) sorption of ambient molecular water from the laboratory atmosphere on hair α-keratin,[16] and (2) exchange of loosely bound structural (hydroxyl and amide) hydrogen between hair keratin and atmospheric vapor.[17,18] Each of these processes has the potential to add non-biological hydrogen or oxygen to hair samples prior to their analysis, compromising the accuracy with which measured hair δ^2H and $\delta^{18}O$ values reflect isotopic signals incorporated during biosynthesis.

EXPERIMENTAL

We conducted three sets of experiments, each involving exposure of hair samples to water vapor of known H- and O-isotope ratio, followed by freeze-drying of the samples and isotope ratio determination. Samples were first weighed (150 ± 15 μg) into pre-combusted Ag-foil capsules. The capsules were then compacted and loaded into plastic sample trays. Exposure of the samples to the waters was achieved by loosely covering the trays and suspending them within sealed, glass desiccators containing ~250 mL of water of known isotopic composition. Two or more isotopically distinct waters were used in each experiment; these exposure treatments were conducted simultaneously, and the desiccators for all treatments were stored in a common, room-temperature environment throughout the exposure period. The loosely covered sample trays were transferred to a sealed container connected to a freeze-drier (Labconco, Kansas City, MO, USA) for sample drying. Samples were then loaded rapidly onto the automated carousel of a ThermoFinnigan thermochemical elemental analyzer (Bremen, Germany), which was evacuated, purged with helium, and opened to the reactor. Following the loading of samples, we monitored the background voltage on masses 28, 29, and 30 until stable, low baseline values were achieved (indicating that little free water vapor was entering the TC-EA system) before beginning the analyses. H- and O-isotope determinations were made by IRMS (ThermoFinnegan DeltaPlus) following pyrolysis and reduction of the resultant H at 1400°C and chromatographic separation of H_2 and CO in a He gas stream. Measured values were calibrated to repeat analyses of an in-house cellulose standard and are reported relative to VSMOW on the VSMOW—SLAP reference scale. Analytical precision, based on the repeated analysis of an unequilibrated powdered keratin standard, was 1.7‰ (δ^2H) and 0.3‰ ($\delta^{18}O$; 1σ, $n = 38$).

In all experiments we used two horse hair samples which had been previously selected and characterized. Both horses were known to have lived in a fixed location (Silver Springs, Florida, hereafter FH; and Provo, Utah, hereafter UH) and to have been fed a relatively homogeneous diet for the last several years. A large sample of hair from the mane of each animal was cleaned of debris and rinsed in 2:1 chloroform/methanol solution to remove lipids.

Measurements of δ^2H and $\delta^{18}O$ in 5 mm segments taken along the length of hair from each sample demonstrated the presence of some intra-hair isotopic variability ($1\sigma = 4$‰ δ^2H and 1.0‰ $\delta^{18}O$ for FH; and 3‰ δ^2H and 0.5‰ $\delta^{18}O$ for UH). Hair samples were processed to achieve homogeneity in one of two ways. First, several grams of each sample were pulverized using a ball mill (Retsch, Newtown, PA, USA), and aliquots of this material were separated for analysis (hereafter: 'pulverized'). Second whole, randomly oriented segments of hair were combined into bundles of 4–6 hair segments, and short (~3 mm) pieces of hair were chopped from these bundles to obtain the necessary amount of sample for analysis (hereafter: 'chopped'). Both homogenization/preparation methods were used in the experiments as indicated below.

Dry-down

A first set of experiments was conducted to determine the relative effect of water sorption and hydrogen exchange on the measured isotope ratios of mammal hair samples. Chopped and pulverized hair samples were exposed to vapor in equilibrium with either light or heavy water (light: $\delta^2H = -116$‰, $\delta^{18}O = -14.8$‰; heavy: $\delta^2H = +192$‰, $\delta^{18}O = +4.9$‰) for a period of 4 days. Previous work has suggested that the hydrogen isotope exchange reaction is quite rapid at room temperature, reaching equilibrium within 1–2 days,[19–21] but times-to-equilibrium as long as 16 days have been reported for feather keratin.[10] Our choice of a 4-day equilibration time was based on the results of two sets of experiments described below. Following the equilibration period, samples were transferred to the freeze-drier system for periods of between 0.5 and 10 days. All samples were transferred simultaneously to the carousel of the TC-EA coupled to the IRMS system and analyzed as described above. An additional set of samples (below referred to as drying time = 0) was transferred directly from the vapor-exposure desiccators to the sample TC-EA carousel and analyzed independently following a brief (~30 min) period of vacuum-drying in the sample carousel.

Forward exchange

A second set of experiments was performed to test the rate at which hydrogen isotope exchange between keratin and ambient water vapor occurs at room temperature. Samples of chopped and pulverized hair were exposed to vapor in equilibrium with either heavy or light water (as above) for periods of between 0.5 and 7 days. All samples were then freeze-dried for a period of 7 days and analyzed as described above.

Additional forward-exchange experiments tested for a dependence of hydrogen isotope exchange rate on atmospheric relative humidity (RH) and on the physical state of the sample capsules during the exchange procedure. For the RH experiments, samples of chopped hair were exposed to vapor in equilibrium with either heavy water (heavy water, above) or a solution of one of three waters (heavy, as above; zero, $\delta^2H = 0$‰, $\delta^{18}O = 0.0$‰; and light, as above) saturated with $CaCl_2$ (isotope ratios for the salt solutions were not determined). The dissolved salt was used to reduce the vapor pressure of the solution, giving an RH of ~41% in the desiccator atmosphere for these 'dry' exposure treatments. Equilibration periods ranged from 66 h to 8 days in length. All samples were transferred to the freeze-drier system for a period of 7 days and analyzed as described above. For the capsule geometry experiment, samples of pulverized FH

were weighed into silver capsules and exchanged with heavy water (7 days; the heavy water used for these experiments had $\delta^2H = +178‰$, $\delta^{18}O = +5.1‰$) and dried (7 days) with or without compacting the sample capsules. Capsules that had not been compacted prior to exchange and drying were compacted at the lab bench immediately following the drying phase and all samples were loaded onto the TC-EA carousel as rapidly as possible and analyzed as described above.

Back-equilibration

A final set of experiments tested the rate at which samples previously equilibrated with heavy water vapor re-equilibrated with water vapor in the laboratory atmosphere. Samples of chopped and pulverized hair were exposed to vapor in equilibrium with either light or heavy water (as above) for a period of 4 days, and then transferred to the freeze-drier system for between 0 and 4 days. One set of samples was loaded in the carousel of the TC-EA coupled to the IRMS system and analyzed immediately, whereas the loosely covered sample trays holding two other sets were placed on the lab bench for 7 or 17 h before being loaded and analyzed.

Data analysis

The standard δ notation for reporting stable isotope ratios ($\delta = (R_{sample}/R_{standard} - 1) \times 1000$, where R is the ratio of the heavy to light isotope) is non-linear with respect to the abundance of either isotopic species. This non-linearity leads to large errors in calculations based on δ values, particularly when they involve large ranges of values (as is the case for most H isotope systems[22]). Although we report our data in δ notation, throughout this work we have used the exact form of all isotope mass-balance equations (i.e. those written in terms of R) and have calculated differences among isotope ratios as ε values ($\varepsilon_{a-b} = ((\delta_a + 1000)/(\delta_b + 1000) - 1) \times 1000$).

We fit the data from our dry-down experiment using an exponential model of the form:

$$\hat{R}(t) = R_{dry} + (R_{wet} - R_{dry})e^{-\lambda t} \quad (1)$$

where $\hat{R}(t)$ is the model-predicted isotope ratio at time t, R_{wet} is the isotope ratio of the sample when wet (i.e. before any drying), R_{dry} is the isotope ratio of the sample when dry, and λ is a first-order rate constant. The assumption that drying is a first-order rate process involving the removal of water with a uniform isotopic composition is implicit in the exponential model, and should be reflected in a relation between reaction progress and time that is linear in semi-log space. We checked this assumption by calculating the reaction progress (P) at time t as:

$$P(t) = \frac{R_m(t) - R_{wet}}{R_{dry} - R_{wet}} \quad (2)$$

where $R_m(t)$ is the measured isotope ratio of the sample (in δ notation) at time t, and plotting $\ln[1 - P(t)]$ against dry-down time. For each experiment, values of R_{wet}, R_{dry}, and λ were chosen to minimize the least-squares difference between measured and model-predicted values.

The mass balance equation:

$$R_{dry} = R_e(F_e) + R_n(1 - F_e) \quad (3)$$

describes the measured hydrogen isotope ratio (R_{dry}) of a dried hair sample in terms of the isotope ratios of its exchangeable (R_e) and non-exchangeable (R_n) hydrogen and the fractional abundance of exchangeable hydrogen (F_e). For our experiments, we assumed that R_e is related to the hydrogen isotope ratio of the treatment waters (R_w) by a constant fractionation factor α_e, where $\alpha_e = R_e/R_w$, and that R_n and F_e are constant for any hair sample regardless of treatment. If we have data from two exchange experiments involving waters with different R_w, substituting $\alpha_e \times R_w$ for R_e in Eqn. (3) and combining the equations for the two experiments gives:

$$\alpha_e F_e = \frac{R_{dry1} - R_{dry2}}{R_{w1} - R_{w2}} \quad (4)$$

where the subscripts 1 and 2 refer to the two experimental treatments. This equation represents the exact isotopic mass-balance for hydrogen isotope exchange, as opposed to the approximate solution (in terms of δ notation) applied in most previous work.[17,18] The exact solution presented in Eqn. (4), however, demonstrates that the experimental system described here does not provide an exact solution for either the fractionation factor α or the fraction of exchangeable hydrogen F_e unless the other parameter is known. Since one of our goals in this study was to estimate F_e for hair keratin, out of necessity we adopted the approximation that $\alpha_e = 1$ (this is the same assumption made implicitly by other authors[17,18]). In natural systems, the value of α is typically close to 1 (i.e. in the range of 0.8–1.2), and so the absolute error imposed on our calculations of F_e is likely to be <20%. If future work is able to provide a measured value of α for the keratin/water exchange reaction, more exact values for F_e can be calculated from our data.

A second set of mass-balance equations can be written to describe the effects of sorbed water on the measured stable isotope ratios of hair samples. For the measured δ^2H of a wet hair sample ($R^{^2H}_{wet}$, as an isotopic ratio) this equation is:

$$R^{^2H}_{wet} = \frac{\alpha_e R^{^2H}_w F^H_e + R^{^2H}_n (1 - F^H_e) + \alpha^{^2H}_s R^{^2H}_w F^H_s}{1 + F^H_s} \quad (5)$$

where $\alpha^{^2H}_s$ is the fractionation factor between sorbed water and the experimental water, as defined above, and F^H_s is the fractional concentration of hydrogen from sorbed water in the sample (i.e. the concentration of hydrogen in sorbed water divided by the total concentration of structural hydrogen in keratin). As for the equation describing H exchange, Eqn. (5) can be solved for a system of two experiments involving waters having different hydrogen isotope ratios only to the point of determining the product ($\alpha^{^2H}_s$)(F^H_s):

$$\alpha^{^2H}_s F^H_s = \frac{R^{^2H}_{wet1} - R^{^2H}_{wet2}}{R^{^2H}_{w1} - R^{^2H}_{w2}} - \alpha_e F_e \quad (6)$$

Because we were able to determine (α_e)(F_e) above, the terms on the right-hand side of the equation are known and estimation of F^H_s only requires the approximation that $\alpha^{^2H}_s \approx 1$.

The equation relating measured oxygen isotope ratios $\left(R^{^{18}O}_{wet}\right)$ to the fraction of oxygen as sorbed water (F^O_s) is

somewhat simpler because no exchangeable oxygen is present in keratin:

$$R_{wet}^{^{18}O} = \frac{R_n^{^{18}O} + \alpha_s^{^{18}O} R_w^{^{18}O} F_s^O}{1 + F_s^O} \quad (7)$$

Again, however, the solution for F_s^O is approximate. Both hydrogen and oxygen isotopes thus provided estimates of the amount of water sorbed to our experimental hair samples. The consistency of these estimates was checked given the expected relationship between F_s^H and F_s^O:

$$\frac{F_s^H}{F_s^O} = 2\frac{C_k^O}{C_k^H} \quad (8)$$

where the mole fractions of hydrogen and oxygen in keratin (C_k^H and C_k^O) are related to F_s^H and F_s^O by $F_s = C_s/C_k$ and $C_s^H = 2 \times C_s^O$. We estimated C_k^O/C_k^H based on wt% H and O estimates for 628 dry hair samples run in our laboratory during the last year. The wt% H and O estimates were derived using a peak-area calibration for our TC-EA/IRMS system based on repeated analyses of a cellulose standard over a range of known weights.

RESULTS

Dry-down

The measured δ^2H and δ^{18}O values for chopped and pulverized hair samples evolved in response to drying (Fig. 1), suggesting that water with hydrogen and oxygen isotope ratios differing from those of the sample was being removed during the drying process. Measured isotope ratios typically changed most rapidly within the first 24 h of drying and reached relatively stable values by day 6. By fitting the measured hair isotope ratios with the exponential model we were able to obtain estimates of the isotope ratio of the dry sample after equilibration with light or heavy water (δ_{dry}), the isotope ratio of wet samples before drying (δ_{wet}), and the rate constant for the drying process (λ) for all but two sets of samples (Table 1). For two sample sets exchanged with the light water, the magnitude of $R_{wet} - R_{dry}$ was too small to allow us to constrain the value of λ. Plotting ln[1 – P(t)] against dry-down time showed that the data did not depart strongly from a linear relationship, confirming that the removal of molecular water from the hair samples is a first-order rate process and suggesting that the isotopic

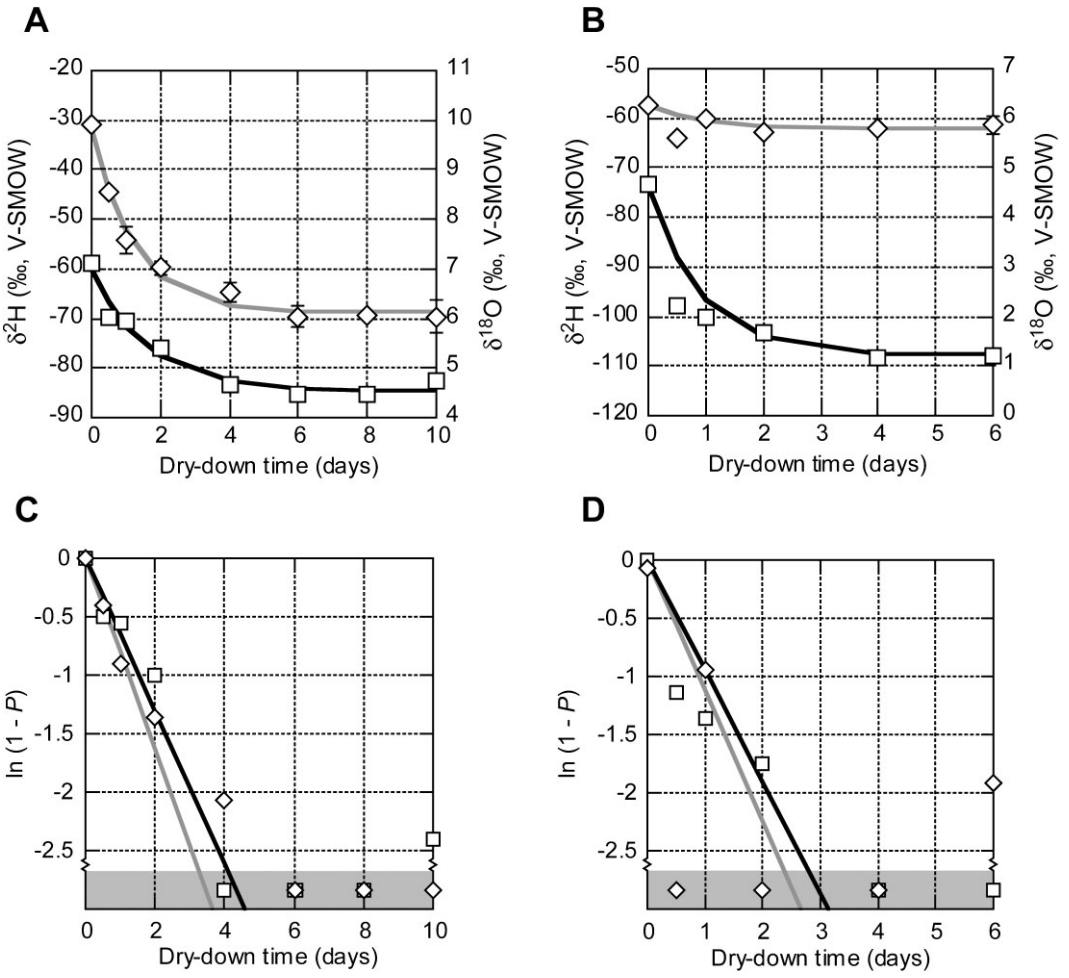

Figure 1. Measured and modeled hydrogen (squares, black line) and oxygen (diamonds, grey line) isotope values of UH hair samples equilibrated with heavy water for 4 days and freeze-dried between 0 and 10 days. Data shown are for chopped (A, C) and pulverized (B, D) samples. (C) and (D) present the isotopic data from (A) and (B) in terms of reaction progress (P). Reaction progress values plotted in the gray band are not different from the values for fully dried samples, within analytical uncertainty. See text for discussion.

Table 1. Model parameters for heavy and light water dry-down experiments

	Hydrogen			Oxygen		
Sample	δ_{dry}	δ_{wet}	λ	δ_{dry}	δ_{wet}	λ
Heavy ($\delta^2H_{wet} = +195‰$, $\delta^{18}O_{wet} = 4.9‰$)						
FH, chopped	−27	−5	1.34	15.1	18.0	1.62
FH, pulverized	−51	−27	0.90	15.2	14.7	0.16
UH, chopped	−85	−60	0.65	6.1	9.8	0.81
UH, pulverized	−108	−74	1.12	5.8	6.3	0.95
Light ($\delta^2H_{wet} = -116‰$, $\delta^{18}O_{wet} = -14.8‰$)						
FH, chopped	−75	−76	n.c.	15.1	16.0	2.07
FH, pulverized	−79	−79	0.79	14.8	14.3	n.c.
UH, chopped	−134	−130	0.71	5.8	7.6	0.60
UH, pulverized	−137	−129	2.11	5.5	7.3	3.69

n.c. = not constrained by available data.

composition of the water being removed did not change significantly through time.

Values of λ were best constrained by the δ^2H values in experiments involving heavy water, in which there were large changes in isotope ratios with time. In these experiments, λ ranged from 0.65 to 1.34 and did not differ systematically among chopped and pulverized hair samples. The average value for λ in these treatments was 1.00, corresponding to a half-life for the drying process of 16.5 h.

Forward exchange

Patterns of isotopic evolution through time were less clear in our forward-exchange experiments (Fig. 2). In most cases, the δ^2H values of samples measured after only 12 h of equilibration showed significant incorporation of the H from the exchange water, reflected as an offset of >20‰ between the heavy and light treatment samples. The δ^2H offset between samples in the two treatments typically reached a maximum within 2–4 days equilibration time, suggesting that exchangeable H equilibrated with the experimental water vapor within that time frame. Maximum values of the δ^2H offset between heavy and light water treatments were consistent in all cases with the δ_{dry} values derived from the dry-down experiments, and indicate that the δ_{dry} values are appropriate for use in calculating estimates of the fraction of exchangeable H in these samples. The measured oxygen isotope difference between samples in the two treatments was typically within ±0.5‰ of zero, confirming that the samples were dry.

In exchange experiments with salt-saturated waters, the measured δ^2H values of hair samples in each treatment were nearly identical following exchange periods of 66, 145 and 192 h (Fig. 3). Significant offsets among the δ^2H values for different treatments were present at 66 h and stable through 192 h, suggesting that complete H exchange between the hair and ambient vapor occurred within 66 h regardless of whether the atmosphere within the exchange desiccator was 'wet' (100% RH) or 'dry' (41% RH). These results suggest that the atmospheric vapor concentration does not strongly affect the H-exchange rate of hair samples, at least when measured over timescales of days.

The δ^2H values of FH samples contained in open or compressed capsules and equilibrated with heavy water were significantly different (Student's t-test, $p = 0.001$). Open-capsule samples had an average δ^2H value of −63‰ ($\sigma = 2.0$, $n = 4$), whereas closed-capsule samples had an average δ^2H value of −56‰ ($\sigma = 1.4$, $n = 4$). These results suggest that capsule geometry was not likely to have had a strong effect on the degree to which hair samples were able to undergo H exchange: samples exchanged in open capsules did not undergo additional exchange due to increased access to experimental vapor. In fact, our results show that open-capsule samples carried less of the heavy H signature of the

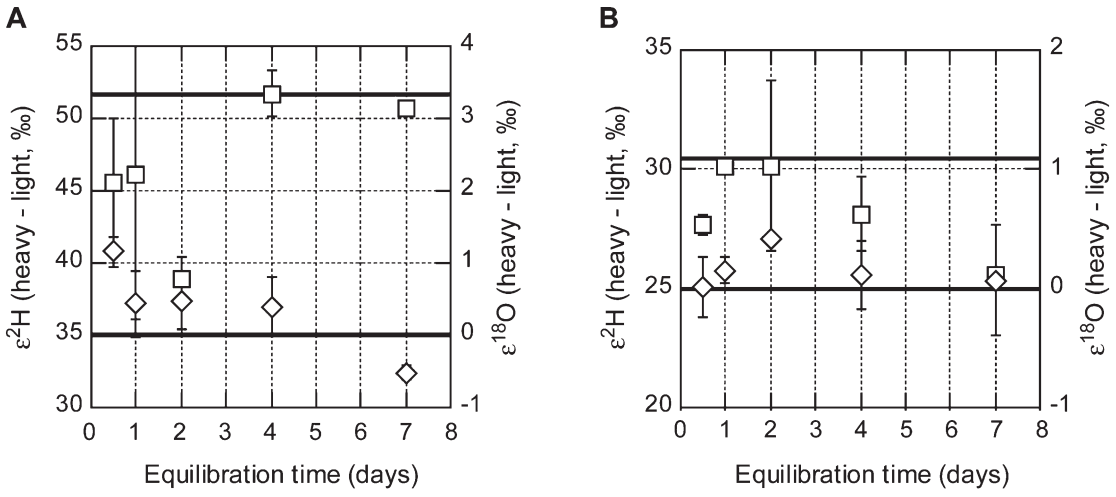

Figure 2. Heavy hydrogen (squares) or oxygen (diamonds) isotope enrichment of FH hair samples equilibrated with heavy water relative to those equilibrated with light water. Equilibration times ranged from 0.5 and 7 days; all samples were dried for 7 days. The equilibrium ε values for H determined in the dry-down experiment and the expected ε value of zero for O are shown by the bold horizontal lines. The maximum ε values for H in the forward-exchange experiments shown are very similar to the those determined in the dry-down experiment, and are achieved within ~4 days, suggesting that the H-exchange process was relatively rapid. (A) Chopped hair samples and (B) pulverized hair samples.

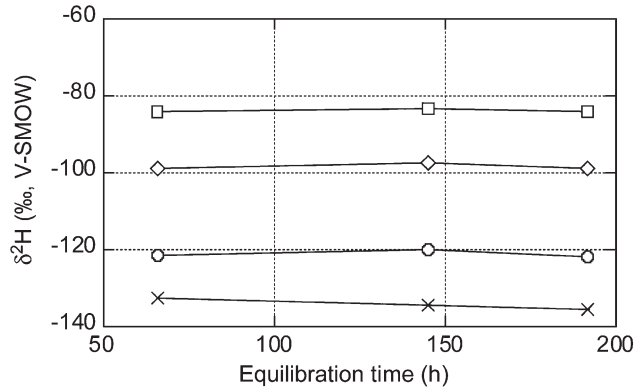

Figure 3. Hydrogen isotope ratios for UH hair samples equilibrated with heavy water or one of three salt-saturated waters and dried for 7 days. Data shown are for chopped hair samples equilibrated with heavy water (squares) and with $CaCl_2$-saturated heavy (diamonds), zero (circles), and light (x's) water. Sample δ^2H values for the different treatments are offset by a consistent amount regardless of treatment length, suggesting that complete H exchange with the treatment waters occurred within 66 h regardless of the vapor pressure of the experimental atmosphere.

exchange vapor at the time of measurement. This may have been the result of small amount of back-exchange with vapor in the laboratory atmosphere during the time required to compact and load the samples onto the TC-EA carousel. Oxygen isotope ratios for the open-capsule and closed-capsule samples were not significantly different (Student's t-test, $p = 0.18$).

Back-equilibration

Samples previously equilibrated with water showed substantial shifts in their δ^2H values after short periods of exposure to the lab atmosphere (Fig. 4), confirming that H exchange occurs under ambient lab conditions over time-scales of minutes to hours. In most cases, the isotopic offset between heavy- and light-equilibrated samples had decreased by 50% or more within 7 h of exposure. After 17 h of exposure, more than 2/3 of the isotopic label was lost. Both wet (no drying) and dry (up to 4 days of drying) samples behaved similarly upon exposure to the lab atmosphere. None of the sample pairs (heavy- and light-equilibrated) had reached identical values, or values that might be in isotopic equilibrium with atmospheric water vapor, within 17 h of back-exchange. In all cases, however, the isotopic offset between back-exchanged samples from the heavy and light water treatments was much less than the value determined above for samples in equilibrium with those waters, indicating a substantial loss of the H-exchange label.

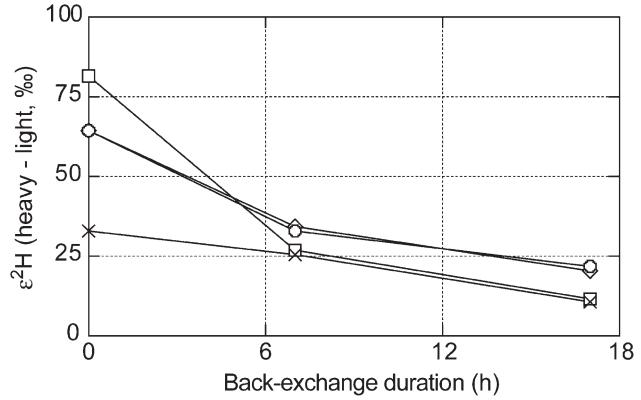

Figure 4. ^2H-enrichment of UH hair samples equilibrated with heavy water relative to those equilibrated with light water following exposure to the lab atmosphere for 0, 7 or 17 h. Data are for chopped hair samples that had been dried for 0 (squares) or 4 (diamonds) days and pulverized samples dried for 0 (circles) or 4 (x's) days. Rapid re-exchange of hydrogen with the lab atmosphere is indicated by substantial convergence of heavy and light treatment δ^2H values within the 17 h test period.

Exchangeable H and sorbed water

We used the estimates of dry and wet sample δ^2H and $\delta^{18}O$ values determined in the dry-down experiments to estimate the contribution of exchangeable H and sorbed water to isotopic measurements of hair samples. The δ^2H_{dry} values for samples equilibrated with heavy water were, without exception, substantially heavier than those for samples equilibrated with light water (Table 1). In contrast, $\delta^{18}O_{dry}$ values for the heavy- and light-equilibrated samples were similar to within 0.4‰. The difference in δ_{dry} values for hydrogen isotopes but not oxygen isotopes is consistent with the exchange of structural hydrogen with water vapor in the equilibration desiccators. Calculated estimates of the fraction of hydrogen that had undergone exchange were remarkably similar for the two horse hair samples when prepared in the same manner, but differed significantly among preparation methods (Table 2). For chopped hair, ~15.9% of the total structural H was estimated to have exchanged with water vapor, whereas, in the pulverized hair samples, ~9.4% of the H exchanged with vapor at room temperature.

Values of δ^2H_{wet} differed significantly for the heavy and light water treatments, and in all cases this difference was greater than the corresponding difference between δ^2H_{dry} values (Table 1). Values of $\delta^{18}O_{wet}$ also differed systematically for the chopped hair samples, but differences between the heavy and light treatments were smaller and less consistent for the pulverized hair sample sets. The increased difference in δ^2H values for the wet samples and the observation of some systematic offset between heavy- and light-equilibrated $\delta^{18}O_{wet}$ values is consistent with the presence of some sorbed molecular water in the samples prior to drying. The concentration of H from sorbed water calculated from the δ^2H_{wet} values was similar for all sample sets and

Table 2. Calculated fractions (mole %) of exchangeable hydrogen and hydrogen and oxygen from sorbed water

	Hydrogen		Oxygen	
Sample	F_e	F_s	F_s	F_s^H/F_s^O
FH, chopped	15.6	7.9	10.1	0.78
FH, pulverized	9.1	7.5	1.9	3.9
UH, chopped	16.2	6.8	11.1	0.61
UH, pulverized	9.6	8.4	n.d.	n.d.

n.d. = not determined.

treatment methods, and averaged 7.7%. Estimated concentrations of O from sorbed water based on $\delta^{18}O_{wet}$ values of chopped hair samples averaged 10.6%, but measurements of the pulverized hair samples provided little or no evidence for sorbed water. The average value of F_s^H/F_s^O for the chopped hair samples was 0.73, similar but not identical to the predicted ratio of 0.53 based on estimated concentrations of H and O in keratin of 5.6 and 23.6% by weight, respectively. Taken together, the data suggest that the wet hair as measured contained between 3 and 4% water by weight.

DISCUSSION

Our experiments verify that hydrogen exchange can have a significant effect on the measured δ^2H values of hair samples. We found that between 9 and 16% of structural H in hair keratin was freely exchangeable with water vapor at room temperature. To put this figure in practical terms, measurements of the same hair sample made in laboratories in Missoula, Montana, and in Houston, Texas, could differ by as much as 11‰ due to exchange with local environmental water vapor alone (using environmental water values of −94‰ for Missoula and −22‰ for Houston; estimated using the Online Isotopes in Precipitation Calculator,[23] following the method of Bowen and Revenaugh[24]). The magnitude of this effect in hair samples is similar to that observed in other keratins and complex organic compounds,[17,18,20] and clearly methodological approaches are needed to correct measured hydrogen isotope ratios of hair for hydrogen exchange.

To this end, we recommend that the approach of Wassenaar and Hobson,[25] involving the calibration of hair standards to determine their δ^2H_n values and the routine analysis of these standards according to the principal of identical treatment (PIT),[26] be adopted for hydrogen isotope analysis of hair. The results presented here for horse hair are encouraging for the application of PIT methods to correct for H exchange in a wide range of mammalian hair samples in that they show that exchange seems to be a rapid and reproducible process. However, they represent only a first step towards understanding the H-exchange properties of hair, and further work to characterize the degree of consistency among hairs from a variety of mammals is required.

We found that the exchange of H between hair keratin and ambient vapor was relatively rapid, and reached equilibrium within 3–4 days regardless of the relative humidity in which the samples were equilibrated. This observation suggests that the physical availability of water molecules to access the sites of H exchange is not a rate-limiting factor in the H-exchange reaction over timescales of days, and is consistent with the very rapid rate at which water molecules penetrate the interior of folded proteins.[27] The geometry of the capsules in which samples were held also seemed to have no effect on the overall amount of exchange through 7 days, indicating that water molecules were able to efficiently access the hair even when it was contained in compacted capsules.

Perhaps the most curious result of our experiments was the finding that the extent of H exchange is greater in whole, chopped hair samples than in mechanically pulverized hair, despite the presumed greater surface area of the pulverized hair. One possible explanation of this result is that δ^2H of sorbed water on these samples changed substantially throughout the drying phase of the experiment, and that continued exchange of H between this sorbed water and structural H of hair affected the chopped and pulverized samples to different degrees. This might be expected, for example, if the rate of drying differed for chopped and pulverized hair. In general, we do not believe that our data support this mechanism. Our dry-down experiment results (Table 1) do not suggest that there is consistent difference in drying rates for chopped and pulverized samples, implying that hair prepared by both methods should have equal opportunity to participate in any continued exchange during the dry-down period. Nor do they provide any evidence that the drying process deviated strongly and systematically from the first-order removal of water with a constant δ^2H, as evidenced by the approximately linear relation between $\ln[1 - P(t)]$ and dry-down time for our experiments (Figs. 1(C) and 1(D)), suggesting that any changes in the δ^2H of sorbed water throughout the drying process were small.

As an alternate mechanism to explain the different extent of H exchange in pulverized and chopped hair, we suggest instead that this observation may be related to changes in the accessibility of H-exchange sites associated with changes in the secondary structure of hair keratin occurring under mechanical stress.[28,29] If correct, this implies that the biochemical structure, rather than the gross physical state, of hair keratin controls the H-exchange reaction. Thus, physical weathering processes that modify the tertiary structure of keratin[30,31] may also affect the H-exchange properties of hair that has been subject to prolonged exposure, for instance, on the head or at archaeological sites. This would reduce the accuracy with which standards of pristine keratin could be used to correct for exchange in weathered samples and perhaps compromise some archeological applications involving the δ^2H of proteins. It is our hope that this possibility will motivate additional research into the effects of prolonged exposure and weathering on H exchange in complex proteins.

Our various results for water sorption on hair were largely consistent with each other, with the exception of estimates derived from the oxygen isotope composition of pulverized hair samples. Although we are not able to provide a conclusive explanation for why these particular estimates differed from the others, we note that there are several reasons to expect the estimates based on O-isotope ratios to be less accurate and precise than those based on δ^2H. In particular, the kinetic fractionation during diffusion of water vapor[32] ($\alpha = 1.025$, $\varepsilon \approx -25$ for H and $\alpha = 1.028$, $\varepsilon \approx -28.5$‰ for O), when compared to the range of water isotope ratios used in this study (311‰ for H and 19.7‰ for O), is much greater for oxygen isotopes than for hydrogen (ratio = 0.08 for H, 1.45 for O). This implies that the estimates of water sorption derived from δ^{18}O data will be much more sensitive to departures from equilibrium vapor balance, such as might occur in association with the partial drying of wet samples loaded into the TC-EA system. Diffusive limitation of the drying process would leave non-uniformly ^{18}O-enriched

'sorbed' water, potentially explaining the inconsistent F_s^O estimates. It is reasonable to expect that this effect would be most severe for pulverized hair samples, since the physical state of these samples allowed their silver foil containers to be tightly compacted.

Despite the relatively low water contents estimated for our samples (3–4 wt%), sorbed water comprised a non-trivial contribution to the measured isotope ratios. Water sorption in similar, better-studied α-keratins, such as wool, can exceed 20% under conditions of high humidity.[33] All of the samples measured in our study, however, experienced some degree of drying once loaded into the sample carousel of the TC-EA system and prior to analysis. For hair samples that are carefully analyzed (i.e. sample carousel purged and carrier gas monitored for the presence of free water) but not thoroughly dried before analysis, our data are likely to provide reasonable estimates of the contribution of sorbed water to measured isotope ratios. Error in δ^2H and δ^{18}O measurements due to the presence of sorbed water is likely to be more significant for hair than for other, more hydrophobic, α-keratins in tissues such as feather or fingernail.

CONCLUSIONS

The results of our experiments confirm that both hydrogen isotope exchange and water sorption have the potential to affect the measured H- and O-isotope ratios of hair. Both processes occur rapidly, reaching equilibrium on a timescale of between one and a few days, and large changes in the measured isotope ratios of hair samples were observed over timescales of hours. Based on our experimental results, we suggest that the following guidelines be incorporated in all treatment protocols for the measurement of hair H- and O-isotope ratios by TC-EA/IRMS:

- Measured H-isotope values should be corrected for exchange using δ^2H_n-calibrated hair standards prepared and analyzed alongside the unknowns. Both samples and standards should be allowed to equilibrate with a uniform moisture source (e.g. the laboratory atmosphere) for at least 4 days prior to drying and analysis. Our results demonstrate that the method of physical processing (e.g. homogenization) can greatly influence the exchange properties of hair, and it is therefore important that standard and sample materials be processed in a uniform manner. Additional work is needed to determine the degree to which exchange properties are consistent among hair from different individuals and species, and whether a single set of hair standards can be developed and applied for analysis of a wide range of hair types.
- Samples should be thoroughly dried (i.e. vacuum-dried for a period of no less than 6 days) prior to analysis, and the procedure for drying explained in published reports.
- Rapid handling of equilibrated, dried samples is needed to minimize back-exchange of hydrogen with and sorption of water from the atmosphere. Although our back-exchange experiments represent an extreme case where samples were strongly out of equilibrium with the laboratory atmosphere, they show that these processes are perceptible over timescales of hours. The most accurate measurements of the biosynthetic H- and O-isotope ratios of hair will thus be made when the exposure time of prepared samples is minimized.

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