WATER SORPTION AND VAPOR-PHASE DEUTERIUM EXCHANGE STUDIES ON METHEMOGLOBIN CC, SC, SS, AS, AND AA

PHILIP J. KILLION *and* BRUCE F. CAMERON

From The New England Institute, Ridgefield, Connecticut 06877, and the Division of Hematology, Department of Medicine, University of Miami, School of Medicine, Miami, Florida 33152. Dr. Cameron's present address is Papanicolaou Cancer Research Institute, Miami, Florida 33136.

ABSTRACT Five hemoglobins whose genetic relationship to one another involves one set of alleles, hemoglobins CC, SC, SS, AS, and AA, were studied in the Met form. Two different investigations were conducted at 28°C on these methemoglobins within a McBain gravimetric sorption system: sorption of H_2O vapor and vapor-phase deuterium-hydrogen exchange. For each of the five samples there was close agreement between the per cent hydration of polar sites as determined from sorption studies and the maximum per cent of labile hydrogens that were exchanged during the vapor-phase deuterium exchange study. Both studies measured a slight increase in the number of polar sites accessible to H_2O or D_2O vapor for those samples in which the substituent in the sixth position from the N-terminus of the two β-chains had a positively charged side chain and a slight decrease for those in which the substituent had a negatively charged side chain. The in-exchange of deuterium for hydrogen occurred at a faster observed rate than the out-exchange of hydrogen for deuterium.

INTRODUCTION

The genetic relationship of hemoglobin SS of sickle-cell anemia to hemoglobin CC (1) was established by Ingram (2) and Hunt (3) when they showed that the glutamic acid in the sixth position from the N-terminus of the two β-chains in normal hemoglobin (hemoglobin AA) had been replaced by valine in hemoglobin SS and lysine in hemoglobin CC. These amino acid replacements result in a difference in net charge of two between sickle-cell hemoglobin (hemoglobin SS) and the other two hemoglobins, and a difference of four between normal hemoglobin (hemoglobin AA) and the abnormal hemoglobin CC.

The above-mentioned work by Hunt and Ingram (3) which characterized the difference in primary structure between the three hemoglobins confirmed a proposal by Ranney (4) that the hemoglobin S and C mutations were allelic. Thus, of the four hemoglobin variants, hemoglobins S, C, D (Los Angeles or Punjab), and E which are found in a very large number of people (5), two result from a

mutation at the same genetic site. This fact becomes very remarkable when one considers that there are 287 other genetic sites involved in the production of the protein chains of hemoglobin.

Therefore, it was of interest to the authors to study these five hemoglobin samples whose genetic relationship to one another involved one set of alleles, and which have considerable clinical importance. The three homozygous samples in which the alleles were identical and resulted in the production of hemoglobins AA, SS, and CC, and the two heterozygous samples in which one of the nonidentical alleles was an S gene (sickle-cell gene) and resulted in the production of hemoglobins SC and AS, were chosen for this study. The one other heterozygous condition which results in the production of hemoglobin AC was not used.

Recently Brausse et al. (6) conducted water adsorption studies on horse hemoglobin at 10.5, 15, 20, and 25°C. In this study it was found that freshly lyophilized material contains 60% Met and 40% oxyhemoglobin. 5 wk later after exposure to a medium degree of hydration, it was found to contain 80% methemoglobin. Therefore, for reasons of stability it was decided to have all the types of human hemoglobin used in this study converted into the Met form, and thus prevent any differences that might result from conversion during the course of plotting an isotherm or during a study of the deuterium-hydrogen exchange reaction from affecting the results. The Met derivative was also chosen because for hemoglobin AA it has been shown to be crystallographically isomorphous with oxyhemoglobin (7).

The water vapor adsorption studies presented below are an expansion on previous work (8) that we have done with the Met form of two human hemoglobins, methemoglobin AA and methemoglobin SS, in one of our interconnected McBain tube sorption systems (9).

The vapor-phase deuterium-hydrogen exchange studies which are also conducted in the McBain sorption system were undertaken to see what further evidence they might provide about the conformation of these proteins and how they might be correlated with the sorption studies.

The existence of vapor-phase deuterium exchange was first inferred by Hnojewyj and Reyerson (10). The method was first used on the globular protein, β-lactoglobulin (11) in which case a little more than 74% of the labile hydrogens were exchanged. This paper reported also that vapor-phase deuterium exchange was reversible. In another paper by Hnojewyj and Reyerson (12) it was reported that lysozyme exchanged 91% of its labile hydrogens. In addition to the protein studies mentioned above this method has been employed by Killion and Reyerson to study DNA (13).

EXPERIMENTAL SECTION

Materials

Samples of blood were obtained from individuals of known genotype as follows: hemoglobins SS, SC, and CC from patients at the University College Hospital, Ibadan, Nigeria; hemoglobin

AS from a patient at Jackson Memorial Hospital, Miami, Fla.; and normal hemoglobin AA from a laboratory technician. All samples were drawn in acid-citrate-dextrose anticoagulant except AA, which was drawn in ethylenediaminetetraacetate (EDTA). Hemolysis and preparation of oxyhemoglobin are as described by Cameron and George (14). The samples from Nigeria were lyophilized as oxyhemoglobin in 0.1 M NaCl and kept frozen except while hand carried to Philadelphia, and thence to Miami. The samples of AA and AS were freshly prepared and oxidized with the SS, SC, and CC samples. Conversion to MetHb was by ferricyanide (14) followed by exhaustive dialysis and lyophilization.

The lyophilized samples of the Met form were hand carried to The New England Institute for analysis within the McBain sorption system.

These lyophilized samples, which were used to load the adsorption vessels, were checked spectrophotometrically and were 100% in the ferric form, without evidence of denaturation. The minor spectral changes previously seen in lyophilized hemoglobin (14) were noted but this is without significance. As lyophilized from distilled water, hemoglobins SS and SC showed a few per cent of the alkaline Met form, consistent with the relative isoelectric points of the respective hemoglobins.

Method

The McBain sorption system (9) within which all the experimental measurements were made had the following construction. Each McBain tube consisted of two water-jacketed cylinders of 4 cm I.D. connected by a glass taper joint. The lower tube in which the sample was suspended was about 70 cm in length and the upper tube about 55 cm. The sorption system differed slightly from the one the authors used previously (8, 13) and contained a total of six McBain tubes which were interconnected by large-bore tubing to vacuum pumps, one of which was an Hg diffusion pump. Suspended in each tube was a quartz spiral spring of the same sensitivity (1 mm/mg) permitting a determination of a weight change of 0.01 mg by a precision cathetometer (described below). The five samples used in this study were held in quartz cylindrical buckets weighing 200 $\pm$50 mg which were hooked onto the spiral springs.

The base weights for the H_2O isotherms and the desorption weights for the vapor-phase deuterium exchange study were determined in a vacuum of less than 10^{-6} mm.

The vapors for the isotherm and exchange experiments came from small reservoirs attached by side arms to the evacuated system. In this sorption system there were two reservoirs containing 99.8% D_2O and two containing water of very low conductivity, obtained by double distillation in a glass system. The various vapor pressures required from the H_2O and D_2O side arms were produced by allowing the vapor to pass through 4 ft of 0.5 mm diameter capillary tubing for very low vapor pressures and 4 ft of 1.0 mm capillary for the higher vapor pressures. A Kontes 600 ml double-scale McLeod gage (Kontes Glass Co., Vineland, N. J.) permitted determinations of pressures below 0.5 mm, while those above that point were measured by sighting a manometer with a special Gaertner cathetometer (Gaertner Scientific Corp., Chicago, Ill.) constructed with an M 912 support yoke, an M 1238 square section, and an M 901 telescope.

A temperature of 28°C was maintained for both the isotherm and vapor-phase exchange studies. A Fisher unitized bath (Fisher Scientific Co., Pittsburgh, Pa.) containing a volume of about 35 liters served to control the temperature of the McBain tubes. The water from the bath was pumped up to a reservoir above the system from which it was fed by gravity through six separate outlets to each of the six water-jacketed McBain tubes. The flow rate through each McBain tube was about 2.5 liters/min.

The vapor-phase exchange was conducted as follows. After the base weights of the samples

were determined by desorption in a vacuum of less than 10^{-6} mm, D_2O or H_2O vapor was shunted very slowly through the capillary tubing described above until the desired pressure was reached. In most cases the desired pressure is 15 mm which is reached in about 75 min for D_2O and 55 min for H_2O. At 15 mm the vapor pressure is more than twice the amount found from isothermal equilibrium studies to produce monolayer coverage at this temperature (8). Very soon after the vapor pressure reached 15 mm (approximately 5–10 min) desorption was commenced very slowly through 0.5 mm capillary tubing at first and then later through 1.0 mm tubing. In about 3 hr the vapor pressure is reduced to 1 mm. After desorbing overnight the capillary tubing was bypassed and evacuation of the system was continued until none of the samples showed any weight loss over a 72 hr period. No more than 7–9 days were required for the samples to reach a constant weight. The above type of operation was repeated as many times as was necessary to reach a weight that showed no increase or decrease upon carrying out an additional adsorption-desorption cycle. The measured weight gain or loss along with an accurate knowledge of the labile hydrogens of the macromolecule permitted calculation of the amount of exchange that had taken place during each cycle.

The labile hydrogens available for vapor-phase deuterium exchange for all the methemoglobins studied were calculated from the sequences presented by Eck and Dayhoff (15). The total number of labile hydrogens calculated for methemoglobin CC, AA, SC, AS, and SS were 936, 934, 934, 933, and 932, respectively.

RESULTS

H_2O Sorption Study at 28°C

After the five human methemoglobin samples were within the interconnected McBain sorption system, desorption was commenced as described above. It required 3–5 wk for the samples to reach a constant weight (Table I).

The isotherm data for the plots shown below were collected and desorption was begun to see if the previously determined base weights were correct. It was observed that four of the samples weighed less than they did during the previous desorption (Table I). This necessitated a second adsorption of H_2O vapor in which the samples were equilibrated under isothermal conditions at 14.5 and 16.4 mm. In the third desorption no further weight losses were observed. It is of interest to note that the three samples containing methemoglobin S had the largest decrease in weight after the first desorption (Table I).

TABLE I

BASE WEIGHT DETERMINATION OF SAMPLES

Sample	Base weight measured during 1st desorption	Base weight measured during 2nd desorption	Weight loss
	mg	*mg*	%
MetHb CC	103.85	103.85	0.00
MetHb AA	102.78	102.73	0.05
MetHb AS	102.84	102.68	0.16
MetHb SC	99.07	98.58	0.50
MetHb SS	98.81	98.42	0.40

The first five isothermal equilibria were determined specifically for the purpose of constructing plots in terms of the BET equation (16)

$$\frac{P/P_0}{h\{1 - (P/P_0)\}} = \frac{1}{h_m C} + \frac{C - 1}{h_m C}\frac{P}{P_0},$$

where P/P_0 is the relative vapor pressure, h is grams of H_2O adsorbed per 100 g of methemoglobin, h_m is grams of adsorbate which produce BET monolayer coverage, and C is a constant related to the heat of adsorption.

We had reported previously (8) that in the case of H_2O vapor adsorption "all points from a relative vapor pressure of 0.05 up to approximately 0.45 fell on a straight line" so these five equilibria were determined within this range. It required from 2 to 4 days to reach equilibrium for each of five relative vapor pressures ranging from approximately 0.11 to 0.40.

Fig. 1 gives a composite of four of the individually constructed BET plots. The monolayer coverages determined for the five samples presented in decreasing order are as follows: 6.01, 5.90, 5.85, 5.80, and 5.78 g/100 g sample, respectively, for methemoglobin CC, SC, SS, AA, and AS.

The next seven isothermal equilibria were determined to provide further information about a change in slope of the straight line portion of adsorption isotherms which we had observed (8) to occur between 14 and 15 mm vapor pressure for several heme proteins. These seven equilibria were established in the range of approximately 13–16.5 mm vapor pressure. Up to a vapor pressure of 15.5 mm,

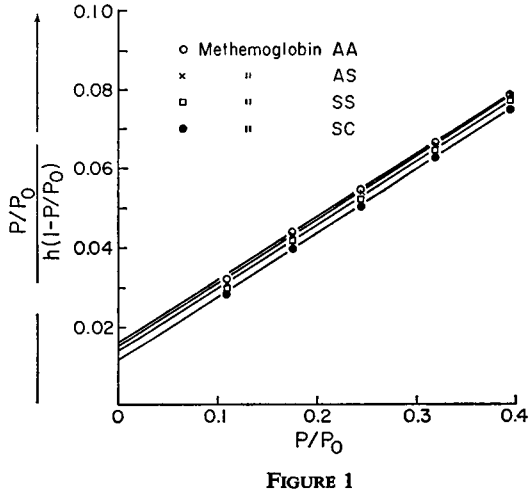

FIGURE 1

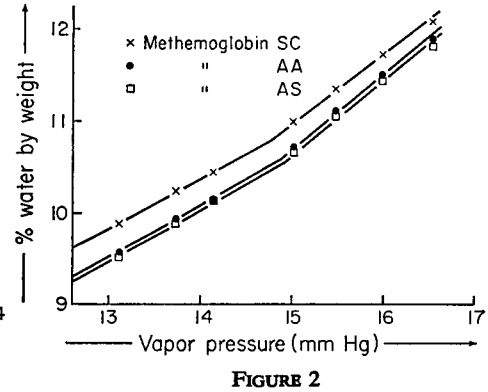

FIGURE 2

FIGURE 1 A composite of four BET plots up to 0.4 relative water vapor pressure for methemoglobins AA, AS, SS, and SC.

FIGURE 2 H_2O adsorption isotherms at 28°C from 13 to 16.5 mm vapor pressure for methemoglobins SC, AA, and AS.

it took no more than 3–4 days to establish equilibrium, but above that vapor pressure it took approximately a week.

Fig. 2 contains three of the plotted isotherms. These three isotherms give a good representation of the measured change in slope. For methemoglobin SC the change in slope took place at approximately 14.8 mm, and for the four other samples it occurred around 14.9 mm vapor pressure.

The grams of H_2O adsorbed per 100 g of sample at the change in slope were 10.94, 10.77, 10.65, 10.58, and 10.55, respectively, for methemoglobin CC, SC, SS, AA, and AS. When these weights for the individual samples are divided by the weight calculated for monolayer coverage, a value of 1.82 is obtained for four of the five samples (MetHb SC = 1.83). The average value of 1.82 layers adsorbed at

the change in slope agrees closely with previously determined (8) averages of 1.75 and 1.86 for this adsorbate on heme proteins.

Vapor-Phase Deuterium Exchange at 28°C

These studies were initiated by conducting a preliminary deuterium-hydrogen exchange in which it was intended to replace approximately one-half of the exchangeable sites with deuterium. This exchange differed from the customary procedure in that it consisted of two very fast adsorption-desorption cycles of D_2O vapor rather than one slower cycle.

On the first adsorption the vapor pressure was allowed to rise to 0.8 mm in the customary manner and then in approximately 5 min it was brought quickly up to 5 mm. Desorption was started immediately and the system remained in the evacuated state over a weekend after which the vapor pressure was 0.07 mm. In the second adsorption, which was carried out more quickly than the first the vapor pressure was raised to 10 mm in about 10 min. Then a second desorption was commenced immediately. This time desorption was continued until constant sample weights could be determined for calculation of deuterium exchange.

The average deuterium exchange for the five samples, expressed as the per cent of total labile hydrogen sites exchanged, was calculated to be 43.2%. As will be shown below, this amounts to 51.2% of the sites that proved to be exchangeable.

Table II reports the data collected during an exhaustive out-exchange of hydrogen for deuterium on these partially deuterated proteins. A total of five adsorption-desorption cycles using H_2O vapor were required to produce complete reprotonation.

This out-exchange was exploratory in nature. Instead of raising the vapor pressure to 15 mm for each cycle as described in the experimental section, vapor pressures ranging from 6 to 15 mm were used during the various cycles (Table II).

Table II shows that the out-exchange reaction took place at a fairly constant rate in which approximately 50% of the sites were exchanged per cycle during the first

TABLE II

PER CENT DEUTERIUM EXCHANGE AT LABILE HYDROGEN SITES AT THE START AND DURING THE COURSE OF OUT-EXCHANGE OF HYDROGEN FOR DEUTERIUM IN FIVE METHEMOGLOBIN SAMPLES IN WHICH APPROXIMATELY ONE-HALF OF THE EXCHANGEABLE SITES CONTAINED DEUTERIUM AT THE START

	Start	1st cycle vapor pressure raised to 6 mm in approx. 32 min	2nd cycle vapor pressure raised to 7 mm in approx. 34 min	3rd cycle vapor pressure raised to 10 mm in approx. 40 min	4th cycle vapor pressure raised to 15 mm in approx. 55 min	5th cycle vapor pressure raised to 15 mm in approx. 55 min
MetHb CC	45.6	21.8	9.92	4.63	0.00	0.0
MetHb SS	42.1	22.4	9.11	6.31	1.40	0.0
MetHb AA	44.9	20.8	8.72	5.37	1.34	0.0
MetHb SC	40.5	18.9	11.2	5.59	0.00	0.0
MetHb AS	43.0	21.5	10.1	7.39	1.34	0.0
Average	43.2	21.1	9.80	5.86	0.82	0.0

TABLE III

PER CENT DEUTERIUM EXCHANGE OF FIVE GENETICALLY RELATED METHEMOGLOBINS

	Adsorption-desorption cycle				
	1st	2nd	3rd	4th	5th
MetHb CC	58.8	77.4	84.7	88.0	88.0
MetHb SC	58.0	75.4	83.1	85.9	85.9
MetHb SS	58.2	74.3	81.3	84.8	84.8
MetHb AA	56.3	73.7	79.1	81.8	81.8
MetHb AS	56.4	73.9	78.6	81.2	81.2
Average	57.5	74.9	81.4	84.3	84.3

three adsorption-desorption cycles. With completion of the third cycle more than 86% of the deuterated sites were reprotonated. The highest vapor pressure was used in the last two cycles, 15 mm, and an increase in the rate of exchange was observed.

A second deuterium exchange study was made to determine the maximum amount of labile hydrogen that is accessible to exchange in these samples and to compare these maximum exchange values with monolayer coverage. Table III reports the data collected during this study. As was observed with the out-exchange reported above, this in-exchange of deuterium for hydrogen occurred at a fairly constant rate during three successive cycles of exchange. The in-exchange was faster than the reverse reaction with an average of 67.4% of the exchangeable sites reacting in each cycle. This left only 3.4% of the accessible sites unexchanged at the end of the third cycle as opposed to 13.6% for the out-exchange.

The fifth adsorption-desorption cycle, in which there was no further exchange, demonstrated that maximum exchange had been reached during the fourth cycle by all of the five methemoglobins.

The last two columns of Table III which report maximum exchange show that most of the labile hydrogens in these methemoglobins were exchanged for deuterium when subject to vapor-phase deuterium exchange. Methemoglobin CC showed the most reactivity by exchanging 88.0% of its labile hydrogens while methemoglobin AS showed the least reactivity by exchanging 81.2% of its labile hydrogens.

The arrangement of the five methemoglobins in order of decreasing reactivity is identical with the arrangement of these samples in order of decreasing monolayer coverage. Table IV presents a comparison of the calculated results from H_2O vapor sorption studies to the measured maximum per cent deuterium exchange. Column two of Table IV reports the BET monolayer coverages. The calculated per cent hydration of polar sites shown in column three were obtained by using the value determined by Brausse et al. (6) of 6.95 g of H_2O/100 g of methemoglobin for the weight required to occupy all polar sites.

For all of the individual samples there is close agreement between the calculated per cent hydration of its polar sites and the per cent of labile sites that were exchanged. Thus, for this type of conformation these two distinctly different types of measurements give similar results.

The arrangement of these five genetically related methemoglobins according to the number of polar sites accessible to H_2O or D_2O vapor as shown in Table IV is of interest. Those containing two segments in which the substituent in the sixth position from the N-terminus of the two β-chains is lysine (MetHb CC and SC)

TABLE IV

COMPARISON OF CALCULATED RESULTS FROM H_2O SORPTION STUDIES TO MAXIMUM PER CENT DEUTERIUM EXCHANGE

Sample	BET monolayer coverage	Calculated % hydration of polar sites from monolayer coverage	Maximum % deuterium-hydrogen exchange at labile hydrogen sites
	g H_2O per 100 g sample		
MetHb CC	6.01	86.47	87.96
MetHb SC	5.90	84.89	85.88
MetHb SS	5.85	84.17	84.82
MetHb AA	5.80	83.45	81.81
MetHb AS	5.78	83.17	81.24
Average	5.87	84.43	84.34

TABLE V

PER CENT DEUTERIUM EXCHANGE AT LABILE HYDROGEN SITES AT THE START AND DURING THE COURSE OF OUT-EXCHANGE OF HYDROGEN FOR DEUTERIUM IN FIVE METHEMOGLOBINS*

	Start	Adsorption-desorption cycle					
		1st	2nd	3rd	4th	5th	6th
MetHb CC	88.0	31.7	15.9	8.60	3.97	2.64	0.0
MetHb SC	85.9	31.4	16.8	8.38	4.19	1.40	0.0
MetHb SS	84.8	30.1	16.1	7.71	4.20	1.40	0.0
MetHb AA	81.8	28.8	14.1	6.71	2.68	0.67	0.0
MetHb AS	81.2	28.2	12.8	6.04	2.69	0.67	0.0
Average	84.3	30.0	15.1	7.49	3.55	1.36	0.0

* Samples in which all of the exchangeable sites contained deuterium at the start.

which has a positively charged polar side chain have more sites accessible to vapor. Those containing two segments in which the substituent in this same position is glutamic acid (MetHb AA and AS) which has a negatively charged polar side chain have less sites accessible to vapor. Methemoglobin SS in which the substituent is valine which has a nonpolar side chain has an intermediate number of sites accessible. It should be noted that although the ratio of S to C in heterozygous hemoglobin SC is one, the ratio of S to A in sickle-cell trait hemoglobin is always less than 1 (17). This may help to explain why the accessibility to vapor of methemoglobin SC is measured to be between homozygous methemoglobins CC and SS while that for methemoglobin AS was not between homozygous methemoglobins SS and AA.

The last hydrogen-deuterium exchange study was made to follow the kinetics of the out-exchange reaction when the starting material has all its accessible labile sites deuterated.

All the data collected during this out-exchange study are shown in Table V. During the first adsorption-desorption cycle the rate of the reaction proved to be fast with 64.4% of the deuterated sites out-exchanged for the average sample. This may be contrasted with the slightly faster rate for the reverse reaction in which approximately 67.4% of the sites exchanged each time on an average during three successive cycles of exchange.

After the first hydrogen-deuterium exchange the rate of the reaction decreased to a slower but steady rate during the next three cycles of exchange in which approximately 50% of the remaining deuterated sites were protonated each time. It should be noted here that the rate of this out-exchange reaction during the second, third, and fourth cycles is similar to the rate of the previous out-exchange reaction on the partially deuterated samples presented in Table II. Thus it may be inferred that when from 40 to 7% of all the labile sites are deuterated the over-all mechanism of vapor-phase exchange of hydrogen for deuterium occurs at a constant rate.

In all three of the exchange reactions presented above, Tables II, III, and V, there was an increase in rate at the very end of the reaction; however, since the amount of material being measured at the end of these reactions is decreasingly small, no firm conclusions about final reaction rates can be drawn.

DISCUSSION

Concurrently in 1950 it was shown by Perutz and Mitchison (18) and Harris (19) that deoxygenated hemoglobin S has a much lower solubility than deoxygenated hemoglobin A or either type of oxyhemoglobin; however, oxygenated hemoglobin S and oxygenated hemoglobin A have the same solubility and the Met derivatives which were chosen by the authors for this study are similar to oxyhemoglobin in solubility. In the case of hemoglobin A both the Met form and oxyhemoglobin have been shown to be crystallographically isomorphous (7).

It was therefore of interest to see if either of the two McBain sorption system methods were capable of measuring any differences that might be due to a change in substituent in this key β-6 position. Genetic mutation has provided in the case of the methemoglobins reported in this study a unique opportunity to study the effects of the substitutions of an amino acid with a positively, negatively, and non-charged (hydrophobic) side chain on a given position in a globular protein. Furthermore it is of importance to consider these more subtle differences before undertaking a study of deoxygenated hemoglobin in which there is such a drastic difference in solubility between hemoglobin S and hemoglobins A and C.

The combination of the results of sorption and deuterium-hydrogen exchange studies at this temperature (28°C) provides some information about the over-all mechanism of adsorption of vapor at the polar site, followed by exchange and then desorption.

The results show that isotopic exchange will occur eventually on all those polar sites that are occupied by the first layer of adsorbate if the adsorbent is subject to repeated exposure of the adsorbate.

Since there are numerous labile hydrogen sites of different reactivity it must be inferred that the time in which the adsorbate is held at the labile site before being desorbed is large in comparison with the time required for the slowest of these exchange reactions to take place. If this were not so it is unlikely that a fairly constant rate of exchange would be observed throughout in-exchange and a good portion of the two out-exchanges.

In the case of in-exchange, the probability that one of the two deuterons of the adsorbate would replace a proton at a labile site was approximately two-thirds during a given adsorption-desorption cycle. The out-exchange is more difficult to interpret because the exhaustively deuterated samples exchanged at a faster initial rate. During the period of constant rate measured for both out-exchanges studied, however, the probability of one of the two protons of the adsorbate replacing a

deuteron at a labile site was approximately one-half during a given adsorption-desorption cycle.

The explanation for the initial change in rate measured during the complete out-exchange of the samples that were approximately 84.3% deuterated at the start of the reaction may be that the rate at which out-exchange occurs is related to the percentage of labile sites that contain deuterium.

Tomita et al. (20) found that the hydrogen bonds in synthetic, helical polypeptides were lengthened by 0.025–0.029 A as a result of deuteration. The helix became elongated along the helix axis without change in helical rotation. Since about 80% of the hemoglobin molecule is helical the more open conformation produced by a high per cent of deuteration might induce the exchange of hydrogen for deuterium to take place at a higher initial rate.

Recent solution studies of deuterium-hydrogen exchange, however, have shown that there is a conformational influence on the chemical exchange rate (21–23) and a solution study on the effects of partial deuteration on the properties of human hemoglobin (24) shows that there is a specific conformational effect on this protein, indicated by a reduction in the cooperativity of oxygen binding by the different subunits.

Since a higher vapor pressure of 15 mm was used in the first cycle of the second out-exchange (Table V) as opposed to 6 mm for the first adsorption-desorption cycle of the first out-exchange (Table II), there is another complication which deserves consideration. The possibility that the second layering of adsorbed H_2O molecules can increase the exchange rate through exchanging with the first layer or by exerting a conformational influence should be investigated.

Some other effects have been demonstrated when deuterium replaces hydrogen, i.e., a reduction in the rate of urea denaturation (25) and a decrease of 11°C in the random coil to helix transition temperature (26). Perhaps the most relevant to protein chemistry is the demonstrated toxic effect of deuterium (27).

The effect of replacement of deuterium for hydrogen, however, did not change the monolayer coverage on three heme proteins in a previous study by the authors (8), nor did it show any difference in the number of polar sites available for deuterium exchange as opposed to those sites available for hydration in the predeuterated samples in this study.

We wish to acknowledge indebtedness to Mr. H. Slominski for the glass blowing so necessary to this research.

This study received partial financial support from the Camille and Henry Dreyfus Foundation.

Received for publication 26 July 1971 and in revised form 3 November 1971.

REFERENCES

1. ITANO, H. A., and J. V. NEEL. 1950. *Proc. Nat. Acad. Sci. U. S. A.* **36**:613.
2. INGRAM, V. M. 1957. *Nature (London)*. **180**:326.

3. HUNT, J. A., and V. M. INGRAM. 1958. *Nature (London)*. **181**:1062.
4. RANNEY, H. M. 1954. *J. Clin. Invest.* **33**:1634.
5. LEHMANN, H., and R. G. HUNTSMAN. 1966. *In* Man's Haemoglobins. North Holland Publishing Co., Amsterdam. 76, 126.
6. BRAUSSE, G., A. MAYER, T. NEDELZKA, P. SCHLECHT, and H. VOGEL. 1968. *J. Phys. Chem.* **72**: 3098.
7. JOPE, H. M., and J. R. P. O'BRIEN. 1949. *In* Hemoglobin. F. J. W. Roughton and J. C. Kendrew, editors. Interscience Publishers Inc., New York. 269.
8. KILLION, P. J., L. H. REYERSON, and B. F. CAMERON. 1970. *J. Colloid Interface Sci.* **34**:495.
9. MCBAIN, J. W., and A. M. BAKR. 1926. *J. Amer. Chem. Soc.* **48**:690.
10. HNOJEWYJ, W. S., and L. H. REYERSON. 1959. *J. Phys. Chem.* **63**:1653.
11. REYERSON, L. H., and W. S. HNOJEWYJ. 1960. *J. Phys. Chem.* **64**:811.
12. HNOJEWYJ, W. S., and L. H. REYERSON. 1961. *J. Phys. Chem.* **65**:1694.
13. KILLION, P. J., and L. H. REYERSON. 1966. *J. Colloid Interface Sci.* **22**:582.
14. CAMERON, B. F., and P. GEORGE. 1969. *Biochim. Biophys. Acta.* **194**:16.
15. ECK, R. V., and M. O. DAYHOFF. 1966. Atlas of Protein Sequence and Structure. The National Biomedical Research Foundation, Silver Springs, Md.
16. BRUNAUER, S., P. H. EMMETT, and E. TELLER. 1938. *J. Amer. Chem. Soc.* **60**:309.
17. INGRAM, V. M. 1963. *In* The Hemoglobins in Genetics and Evolution. Columbia University Press, New York. 53.
18. PERUTZ, M. F., and J. M. MITCHISON. 1950. *Nature (London)*. **166**:677.
19. HARRIS, J. W. 1950. *Proc. Soc. Exp. Biol. Med.* **75**:197.
20. TOMITA, K. I., A. RICH, C. DELOZE, and E. R. BLOUT. 1962. *J. Mol. Biol.* **4**:83.
21. KLOTZ, I. M., and D. D. MUELLER. 1969. *Biochemistry.* **8**:12.
22. WOODWARD, C. K., and A. ROSENBERG. 1970. *Proc. Nat. Acad. Sci. U. S. A.* **66**:1067.
23. MILLER, W. G. 1970. *Biochemistry.* **9**:4921.
24. TOMITA, S., and A. RIGGS. 1970. *J. Biol. Chem.* **245**:3104.
25. MAYBURY, R. H., and J. J. KATZ. 1956. *Nature (London)*. **177**:629.
26. CALVIN, M., J. HERMANS, JR., and H. A. SCHERAGA. 1959. *J. Amer. Chem. Soc.* **81**:5048.
27. THOMSON, J. F. 1963. Biological Effects of Deuterium. The Macmillan Company, New York.