6-1962

Reversible Changes in the NEM-reactive--SH Groups of Hemoglobin on Oxygenation-deoxygenation

Samuel A. Morell
Marquette University

Peg Hoffman
Milwaukee Blood Center

Valborg E. Ayers
Milwaukee Blood Center

Fumito Taketa
Milwaukee Blood Center

REVERSIBLE CHANGES IN THE NEM-REACTIVE −SH GROUPS OF HEMOGLOBIN ON OXYGENATION-DEOXYGENATION†*

BY SAMUEL A. MORELL, PEG HOFFMAN, VALBORG E. AYERS, AND FUMITO TAKETA

DEPARTMENTS OF BIOCHEMISTRY, MILWAUKEE BLOOD CENTER, INC., AND MARQUETTE UNIVERSITY SCHOOL OF MEDICINE, MILWAUKEE, WISCONSIN

Communicated by Linus Pauling, April 17, 1962

In a previous communication the reaction of N-ethylmaleimide (NEM) with the thiols of intact human erythrocytes was described.[1] The principal sites of reaction are the β-chains of hemoglobin[2] and intracellular glutathione. Average values of 2.35 and 0.45 equivalents of HbSH and GSH respectively per mole of hemoglobin have been found for normal red cells. The conditions used were NEM/Hb = 8, 1 hr, 25°C, pH 6.8. NEM is relatively unstable above pH 7 and reacts readily with thiols at pH 6.8.[3] As shown in Figure 1, the uptake of NEM by both erythrocytes and dialyzed hemolysates, measured by decrease in absorption at 300 mμ, is rapid during the first 30 minutes and very slow after one hour. The reagent exhibits a relatively high degree of specificity in reacting rapidly with −SH groups.[3-8] No significant amounts of NEM are bound when the −SH groups of hemoglobin are alkylated[8] and the measurement of −SH by amperometric titration has been correlated with OD_{300}.[2] Reactions with other sites on the protein, as well as the imidazole-catalyzed polymerization of NEM,[9] are apparently secondary. Correlations of OD_{300} with S-cysteinosuccinic acid formed on hydrolysis of NEM-treated proteins also indicate that the rapid reaction of NEM with proteins is limited to condensation with −SH groups.[7, 10]

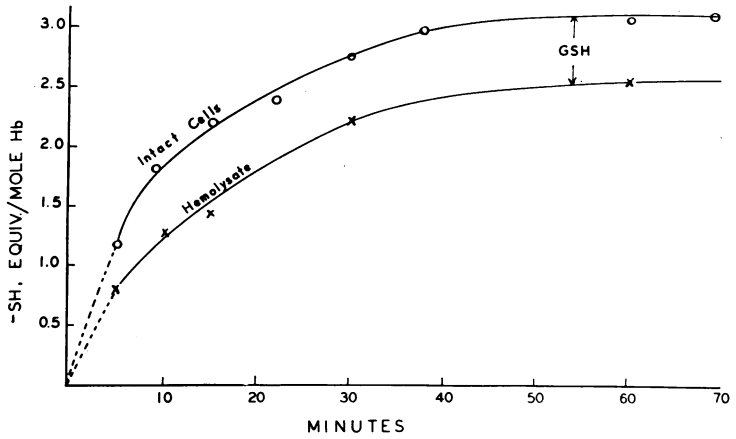

FIG. 1.—Rate of NEM-reaction with intact erythrocytes and dialyzed stroma-free hemolysates. NEM 0.002 *M*, pH 6.8, 23 ± 1°C, molar ratio NEM/Hb = 8. See Table 1 for procedure and calculation of equiv-SH/mole Hb, uncorrected for GSH.

The purpose of the present communication is to report evidence that the reactive −SH groups of hemoglobin undergo reversible changes on oxygenation-deoxygenation. As measured by reaction of NEM with erythrocytes for one hr at pH 6.8, the number of −SH groups decreased on deoxygenation from about 2 (2.3 ± 0.2) to

TABLE 1

DECREASE IN THE NEM-REACTIVE −SH GROUPS OF HEMOGLOBIN ON DEOXYGENATING ERYTHROCYTES

	Blank	Oxygenated			Deoxygenated		
		A_0	B_0	C_0	A_{30}	B_{30}	C_{30}
ml buffer, pH 6.8[11]	5	3	4	4	3	4	4
ml RBC suspn	0	1	0	1	1	0	1
30 min, 23° C						gas with N_2	
ml NEM, 0.01 *M*, pH 6.8	0	1	1	0	1	1	0
OD_{300}		0.423	0.601	0.041	0.539	0.601	0.045
ΔOD_{300}		0.219			0.107		
equiv (HbSH + GSH)/ mole Hb		2.71			1.32		
equiv −SH/mole Hb		2.23			0.84		

A washed suspension of human erythrocytes (hematocrit, 25.0%; Hb, 8.6%; GSH, 19.2 mg %; moles GSH/mole Hb, 0.48) was reacted with an excess of NEM before (A_0 tube) and after (A_{30} tube) deoxygenation with nitrogen for 30 min. Subscripts refer to minutes of bubbling nitrogen through the solutions (1 mm I.D. capillary, 200 ± 25 bubbles/min). The NEM reaction was conducted in stoppered tubes and all transfers were made under nitrogen. After 1 hr at 25°C, pH 6.8, the tubes were immersed in ice and centrifuged at 4°C (stoppered). Aliquots of the clear supernates (negligible hemolysis) were mixed with equal volumes of cold 10% perchloric acid, after which precautions regarding reoxygenation were no longer necessary. After centrifuging, the supernates were filtered through Whatman #44 and read in 1 cm cells at 300 mμ against the blank. The buffer blanks, the NEM tubes (B) and the RBC blanks (C tubes) were treated in an identical manner to obtain $OD_{300} = OD_{300}$ tubes B − (A − C). The molar absorbancy of NEM at 300 mμ, pH 6.8, is 620[4] and an assumed MW of 66,000 for hemoglobin[12] was used to calculate: equiv − SH/mole Hb = (OD_{300}) (66000)/(620) (%Hb) − GSH/mole Hb.

1 (0.9 ± 0.2) equivalents per mole of hemoglobin. The decrease was fully reversible on reoxygenation. On varying the time of reaction with NEM from 15 min to 24 hr, or the NEM/Hb ratio from 3 to 30, the decrease in −SH on deoxygenation remained practically constant, 1.2 ± 0.2. This decrease, referred to as "the Δ-SH reaction," was also demonstrated in dialyzed hemolysates. The pH optimum for the reaction was found to be 6.8–7.0, Δ-SH being reduced about 30 per cent at pH 6.1 and at 7.3.

The decrease in reactive −SH groups of hemoglobin on deoxygenating human erythrocytes is shown in Table 1. On bubbling nitrogen through a washed cell suspension for 30 min at 25°C and pH 6.8, the reactive −SH groups decreased from 2.23 to 0.84 equivalents per mole of hemoglobin, 62.3 per cent. A similar experiment was conducted on a dialyzed hemolysate, −SH/mole Hb decreasing from 2.33 to 1.13, 51.9 per cent.

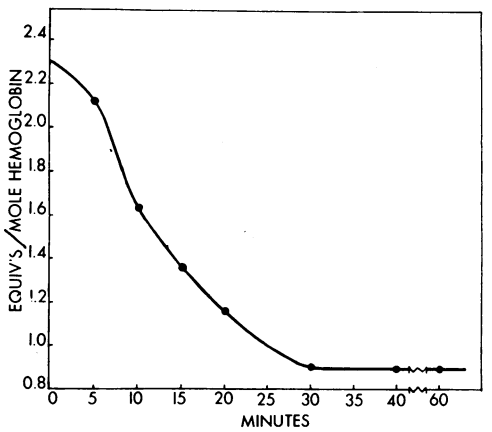

FIG. 2.—Decrease in the NEM-reactive -SH groups of hemoglobin on deoxygenating erythrocytes by gassing with nitrogen for 5–60 min. Data plotted from columns 1 and 3 of Table 2.

The effect of oxygen saturation on the magnitude of Δ-SH and the reversibility of the reaction are shown in Table 2. The data are plotted in Figures 2 and 3 where it may be seen that (*a*) the rate of change of −SH is greatest during the first 10 min of deoxygenation, corresponding to the approximate range of normal respiration, 65–95 per cent saturation;[14] (*b*) in this range of oxygenation where approximately 1 mole O_2/mole Hb is exchanged reversibly, Δ-SH amounts to 0.7 equiv/mole Hb, whereas for the entire range of oxygenation, involving 4 moles of O_2, Δ-SH increases to 1.4; and (*c*) the sigmoid shape of the curve relating the

TABLE 2

REVERSIBLE CHANGES IN THE NEM-REACTIVE −SH GROUPS OF HEMOGLOBIN ON OXYGENATION-DEOXYGENATION OF ERYTHROCYTES

N_2 thru RBC (min)	O_2 Saturation* (%)	−SH per Mole Hb (equiv)	Δ-SH Deoxygn (equiv)	Δ-SH Reoxygn (equiv)	Recovery −SH + Δ-SH / Reoxygn / 2.31 (%)	Δ-SH 0 ⇄ % Satn (equiv)
0	100	2.31				1.40†
5	85	2.14	0.17	0.10	97.0	1.23
10	70	1.68	0.63	0.81	107.8	0.77
15	55	1.38	0.93	0.82	95.2	0.47
20	40	1.19	1.12	1.06	97.4	0.28
30	10	0.92	1.39	1.22	92.6	0.01
40	0	0.94	1.37†	1.39	100.9	
60	0	0.89	1.42†	1.29	94.4	

* Approximated from OD 650/805 at 0, 5, 30 and 60 min N_2.[13]
† Δ − SH on compete deoxygenation = 1.40 (average of 1.37 and 1.42 at 40 and 60 min).

A series of siliconized 15 ml graduated centrifuge tubes was set up for deoxygenation by bubbling with nitrogen. The procedure was identical to that described in Table 1, using either NEM ("A" and "B" tubes) or pH 6.8 buffer ("C" tubes). After removing the aliquots for mixing with 10% perchloric acid to obtain Δ-SH Deoxygn, each tube was immediately stoppered for subsequent reoxygenation ("A" tubes) or analysis[13] for % oxygen saturation ("C" tubes). Prior to reoxygenation, the excess NEM was removed by first aspirating the remaining supernate, under nitrogen, and then washing the cells twice with pH 6.8 buffer (tubes stoppered during centrifuging). After adjusting with buffer to 4 ml, air was bubbled rapidly through the samples for four min. Reaction with 1 ml 0.01 *M* NEM at pH 6.8 for one hr was then repeated to obtain Δ-SH Reoxygn.

progressive reversible change in Δ-SH as a function of the degree of oxygenation simulates that of the oxygen dissociation curve of hemoglobin.

Data relating the NEM-reactive −SH groups of human hemoglobin to the oxygenation process are presently conflicting. Benesch and Benesch[8] report that reduced as well as fully oxygenated hemoglobin binds the same amount of NEM, 2.5 moles in 30 min at pH 7.3, NEM/Hb = 5. Riggs[2] however reports a substantial decrease on deoxygenation, 61 ± 16 per cent in 2 hr at pH 6.2 to 7.6, NEM/Hb = 3. Since 2 hr were required for reduced hemoglobin to exhibit the same extent of NEM-binding as oxyhemoglobin exhibited in 15 min, Riggs interpreted this difference as a rate effect. Only one reaction time however, 2 hr, was reported for reduced hemoglobin. In Figure 4 the effect of time on the reaction of NEM with both oxygenated and reduced hemoglobin is shown. Δ-SH remained constant, 1.1 ± 0.1 equiv./mole Hb, over the 24 hr period studied. Although we have confirmed Riggs' observation that reduced and oxyhemoglobin bind the same amount of NEM in 2 hr and 15 min respectively (1.5 ± 0.1), it appears that one of the two NEM reactive −SH groups is masked on deoxygenation.

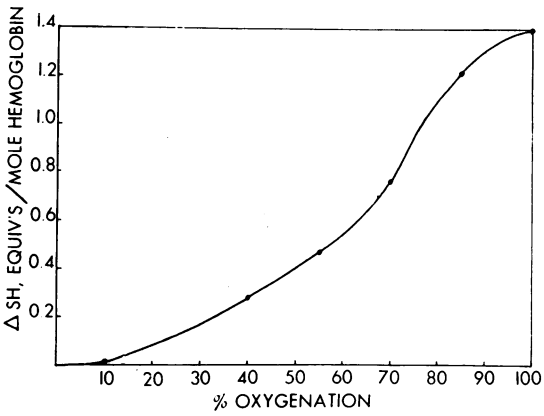

FIG. 3.—Reversible changes in the NEM-reactive −SH groups of hemoglobin on oxygenation-deoxygenation of erythrocytes at pH 6.8. Data for Δ-SH versus % oxygenation plotted from columns 2 and 7 of Table 2.

Evidence for a functional relationship between the sulfhydryl groups of human

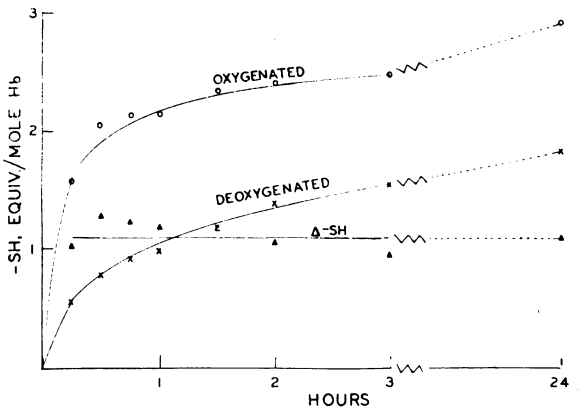

FIG. 4.—Rate of NEM-reaction with oxygenated and deoxygenated erythrocytes. Deoxygenation by gassing with nitrogen for 40 min. NEM 0.002 *M*, pH 6.8, 23 ± 1°C, molar ratio NEM/Hb = 8 ± 1. See Table 1 for procedure and calculation of equiv -SH/mole Hb.

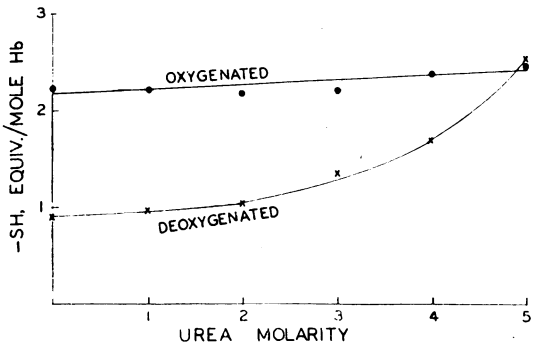

FIG. 5.—Effect of urea on the NEM-reactive -SH groups of oxygenated and deoxygenated hemoglobin. Deoxygenation by gassing with nitrogen for 50 min. 1 ml of dialyzed stroma-free hemolysate (1.1 ± 0.2 × 10^{-3} *M* Hb) in "A" and "C" tubes; NEM 0.002 *M*, pH 6.8, 23 ± 1°C, molar ratio NEM/Hb = 9 ± 2. See Table 1 for procedure and calculation of equiv-SH/mole Hb.

hemoglobin and the oxygenation process has been reported by Riggs.[2] He proposed that the Bohr effect is made up of contributions from at least two different groups and suggested that on oxygenation a hydrogen bond is broken between a β-chain −SH group and an oxygenation-linked acid group. In accordance with this hypothesis, as shown in Figure 5, Δ-SH is reduced to zero in 5 molar urea. The β-chain cysteines which contain the reactive −SH groups are adjacent to histidine residues[15] and H-bonding of these thiol-imidazole groups may be related to the Δ-SH reaction. We have found however that the effect of pH on Δ-SH differs from that reported for the Bohr effect. The pH optimum for $\Delta\text{-H}^+$ is 7.3,[8, 16] whereas that for Δ-SH is 6.8–7.0; and at pH 6.1, where $\Delta\text{-H}^+$ is reduced to zero,[16] Δ-SH is reduced by only about 30 per cent. The objective of our present investigations is to determine whether a functional or a coincidental relationship exists between the Δ-SH reaction of intact erythrocytes and that portion of the Bohr effect, the "isohydric shift," which participates in normal respiration.

We are indebted to S. Raymond Gambino and R. Caseo, St. Luke's Hospital, Milwaukee, Wisconsin, for their cooperation in the measurement of oxygen saturation; to T. J. Greenwalt for his interest and helpful suggestions; and to M. Moorman and M. Jaeckle for their technical assistance.

* N-ethylmaleimide.

† This investigation was supported by a research grant from the National Heart Institute, United States Public Health Service (Grant No. H-4965).

[1] Morell, S. A., V. E. Ayers, and T. J. Greenwalt, *Federation Proc.*, **18**, 290 (1959).

[2] Riggs, A., *J. Biol. Chem.*, **236**, 1948 (1961).

[3] Gregory, J. D., *J. Am. Chem. Soc.*, **77**, 3922 (1955).

[4] Alexander, N. M., *Anal. Chem.*, **30**, 1292 (1958).
[5] Roberts, E., and G. Rouser, *Anal. Chem.*, **30**, 1291 (1958).
[6] Benesch, R., R. E. Benesch, M. Gutcho and L. Laufer, *Science*, **123**, 981 (1956).
[7] Leslie, J., D. L. Williams, and G. Gorin, *Anal. Biochem.*, **3**, 257 (1962).
[8] Benesch, R. and R. E. Benesch, *J. Biol. Chem.*, **236**, 405 (1961).
[9] Smyth, D. G., A. Nagamatsu, and J. S. Fruton, *J. Am. Chem. Soc.*, **82**, 4600 (1960).
[10] Riehm, J. P., and J. C. Speck, *Am. Chem. Soc., Div. Biol. Chem., Abstracts*, p. 34 C, Chicago, Sept. 3–8 (1961).
[11] Hendry, E. B., *Clin. Chem.*, **7**, 156 (1961).
[12] Drabkin, D. L., *Federation Proc.*, **16**, 740 (1957).
[13] Deibler, G. E., M. S. Holmes, P. L. Campbell, and J. Gans, *J. Appl. Physiol.*, **14**, 133 (1959).
[14] White, A., P. Handler, E. L. Smith, and D. Stetten, *Principles of Biochemistry* (2d ed.; New York: McGraw-Hill Book Co. Inc., 1959), p. 666.
[15] Goldstein, J., G. Guidottig, W. Konigsberg, and R. J. Hill, *J. Biol. Chem.*, **236**, PC 77 (1961).
[16] Wyman, J., Jr., *J. Biol. Chem.*, **127**, 581 (1939).

BREAKDOWN OF MESSENGER RNA DURING IN VITRO AMINO ACID INCORPORATION INTO PROTEINS

BY A. TISSIÈRES* AND J. D. WATSON

THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY

Communicated by John T. Edsall, April 3, 1962

Messenger RNA (mRNA) which carries information for the synthesis of specific proteins from DNA to ribosomes, unlike ribosomal and transfer RNAs, is metabolically unstable.[1–3] Thus a single mRNA molecule, attached to a ribosome, serves to make possibly one, or in any case a limited number of protein molecules. It is then broken down, while new mRNA molecules, made on DNA templates, function for the synthesis of new proteins.

There are now indications that mRNA is unstable in cell-free extracts and is broken down to acid soluble fragments.[4, 5] In the work presented here, the conditions for this *in vitro* breakdown were studied. The results show that for mRNA breakdown to occur, both ribosomes and supernatant are necessary. Moreover the rate of breakdown is greatly increased by the addition of ATP and an ATP generating system.

Material and Methods.—1. *C^{14} uracil labeling of mRNA:* The pyrimidine requiring strain of *E. coli* B 148 was cultivated, and the C^{14} uracil pulse labeling of T_2 mRNA were done, essentially as previously described,[5] with the exception that the cells were not starved for uracil before labeling. Growth was followed by optical density measurements, and C^{14} uracil was added shortly before exhaustion of the unlabeled uracil present in the medium (4 μg per ml, allowing growth to about 5×10^8 cells per ml). The cells, washed twice in 0.005 *M* Tris-HCl pH 7.3 and 0.001 *M* Mg^{++}, were kept frozen.

2. *Preparation of cell-free extracts, ribosomes and supernatant: E. coli B* was cultivated and washed, and the crude cell-free extract was made by grinding with alumina and extracting with 3 volumes of 0.005 *M* Tris-HCl pH 7.3 and 0.01 *M* Mg^{++}, containing 5 μg deoxyribonuclease (DNase) per ml as described elsewhere.[6] The washed cells could be kept frozen. In some experiments the extracts were fractionated into ribosomes and supernatant by 2 hr centrifugation at 100,000*g*. The ribosomes were washed twice in at least twice the original volume of the extract of Tris-0.01 *M* Mg^{++} mixture. The upper $^2/_3$ of the supernatant was recentrifuged for 2 hr at