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Research Article

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Oxygen Equilibria of Hemoglobin A_2 and Hemoglobin Lepore*

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Human hemoglobin provides a model for studies concerned with the relationships of structure and biologic function of proteins. Older evidence for conformational differences between oxygenated and deoxygenated normal hemoglobin (1) has recently been confirmed and extended by X-ray crystallographic studies (2) and by comparison of the dissociation (3) and of the hybridization (4) of the oxygenated and deoxygenated pigments.

Normal and abnormal human hemoglobins have been utilized in the past by other workers for investigation of relationships between structure and oxygen equilibria. Some of these observations, grouped on the basis of the structure of the hemoglobins studied, are briefly cited below.

1) Normal human hemoglobins A ($\alpha_2^A \beta_2^A$), F ($\alpha_2^A \gamma_2^F$), and A_2 ($\alpha_2^A \delta_2^{A_2}$) are tetramers containing apparently identical α^A polypeptide chains, but differing in the non-alpha chains. Extensive studies of the oxygen equilibria of hemolysates prepared from the erythrocytes of normal subjects and of normal erythrocytes, containing almost entirely hemoglobin A, provide values with which data on other human hemoglobins may be compared. The increased oxygen affinity observed in the whole blood of newborn infants (5, 6) cannot be due solely to the presence of hemoglobin F, since solutions of hemoglobin F and of hemoglobin A exhibit the same oxygen dissociation curves (7, 8). Furthermore, in the studies of Schruefer, Heller, Battaglia, and Hellegers (8), the oxygen equilibria of erythrocytes obtained from an adult in whom hemoglobin F comprised 69% of the total pigment resembled the oxygen equilibria of normal adult blood rather than that of cord blood. These workers (8) suggested that differences between the fetal and adult red cell other than the type of hemoglobin must be concerned in the oxygenation function of whole blood obtained from newborn infants.

Although hemolysates containing large proportions of hemoglobins F or A can be studied directly, isolation procedures must be utilized to achieve appropriate concentrations of some of the minor components. For example, since hemoglobin A_2 ($\alpha_2^A \delta_2^{A_2}$) accounts for only 5 or 6% of the hemoglobin of subjects with thalassemia minor, for functional studies at least partial isolation of hemoglobin A_2 is required. Rossi-Fanelli, Antonini, Benerecetti, and De Marco (9) isolated hemoglobins A and A_2 electrophoretically and found no significant difference in the oxygen dissociation curves. Meyering, Israels, Sebens, and Huisman (10) and Huisman, Dozy, Nechtman, and Thompson (11), however, employed column chromatography for separations and found that hemoglobin A_2 exhibited increased oxygen affinity as compared with hemoglobin A.

2) Abnormal hemoglobins with single amino acid substitutions. The functions of some of the abnormal hemoglobins that result from single amino acid substitutions in one pair of polypeptide chains have been studied. Among these are β-chain variants, hemoglobin S ($\alpha_2^A \beta_2^{6\,val}$), hemoglobin C ($\alpha_2^A \beta_2^{6\,lys}$), hemoglobin D^β, and an α-chain variant, hemoglobin I. Although decreased oxygen affinity has been observed in whole blood from patients with sickle cell anemia (12–15), data concerning the oxygen affinity of solutions of hemoglobins S are conflicting (16). It seems probable, however, that large differences in

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oxygen affinity do not exist for hemolysates of hemoglobins A and S (8, 17, 18). No difference in the oxygen affinities of hemoglobins C and A has been observed in two studies (4, 18), but small increases in oxygen affinity for two hemoglobin D variants have been reported (18). Hemoglobin I, an α-chain variant, had a normal oxygen equilibrium (4). In the M hemoglobins in which oxidized heme iron is present in either the α or β polypeptide chains, there is obvious interference with the oxygenation function; Kiese, Kurz, and Schneider (19) have reported a case in which enzymatic reduction of hemoglobin M yielded a hemoglobin with a normal oxygen affinity. Reissmann, Ruth, and Nomura (20) described an unusual abnormal hemoglobin with a low oxygen affinity; no structural studies of the pigment were included in the report.

3) Tetramers of one type of polypeptide chain. Studies of functional properties of hemoglobins that are tetramers of one variety of polypeptide chain, i.e., H (β_4^A) and Bart's (γ_4^F), have disclosed striking abnormalities. Both hemoglobin H (21) and Bart's (22) exhibit extraordinarily high oxygen affinities and absence of heme-heme interaction, and neither has a significant Bohr effect. It has therefore been suggested that interactions between α and β chains are necessary for these three aspects of hemoglobin function (21).

4) Hemoglobin Lepore. The structure of hemoglobin Lepore, as elucidated by Baglioni (23), represents a hitherto unrecognized class of hemoglobin abnormalities: normal α chains combined with chains that are products of unequal crossing over in the linked δ-β locus. Thus hemoglobin Lepore contains apparently normal α^A chains and non-alpha chains, which include initial peptides of δ chains and the remaining peptides from the β-chain sequence. Hemoglobin Lepore may be designated α_2^A x^s, where x represents the chain resulting from the crossing over. Since crossing over may occur at different sites, different examples of hemoglobin Lepore might exhibit different lengths of δ (or β) sequence. Huisman, Still, and Nechtman noted an increased oxygen affinity in an example of hemoglobin Lepore (18).

Thus, apart from certain special cases, e.g., the hemoglobin studied by Reissmann and coworkers (20), hemoglobins that are tetramers containing two α chains linked to two β or to two γ chains exhibit nearly the same oxygen affinity as does hemoglobin A. Data concerning the oxygen affinity of hemoglobin A_2 are conflicting (9–11).

The present studies were undertaken to reinvestigate the oxygen function of hemoglobin A_2 and to study the functional properties of another example of hemoglobin Lepore.

TABLE I

Data from which the statistical analyses were calculated

Normal whole hemolysate		Whole hemolysate containing hemoglobin Lepore		Hemoglobin A concentration equivalent to hemoglobin Lepore		Isolated hemoglobin Lepore		Hemoglobin A concentration equivalent to hemoglobin A_2		Isolated hemoglobin A_2	
log P_{50}	pH	log P_{50}	pH	log P_{50}	pH	log P_{50}	pH	log P_{50}	pH	log P_{50}	pH
0.903	7.07	0.875	7.07	0.826	7.00	0.740	6.82	0.813	7.02	0.820	7.00
0.940	7.07	0.903	7.07	0.833	7.00	0.742	6.82	0.820	7.02	0.839	7.00
0.813	7.20	0.839	7.19	0.820	7.00	0.663	7.00	0.699	7.20	0.732	7.19
0.813	7.20	0.839	7.19	0.740	7.18	0.690	7.00	0.708	7.20	0.716	7.19
0.690	7.46	0.681	7.44	0.763	7.18	0.699	7.02	0.623	7.40	0.748	7.21
0.690	7.46	0.681	7.44	0.778	7.22	0.556	7.10	0.623	7.40	0.748	7.21
0.586	7.62	0.550	7.62	0.732	7.22	0.556	7.10	0.544	7.50	0.580	7.42
0.544	7.63	0.538	7.62	0.568	7.44	0.602	7.20	0.544	7.50	0.574	7.42
0.544	7.63			0.586	7.44	0.602	7.20			0.525	7.55
				0.519	7.57	0.643	7.22			0.519	7.55
				0.525	7.57	0.643	7.22				
						0.505	7.38				
						0.512	7.38				
						0.498	7.40				
						0.512	7.40				
						0.380	7.58				
						0.423	7.58				

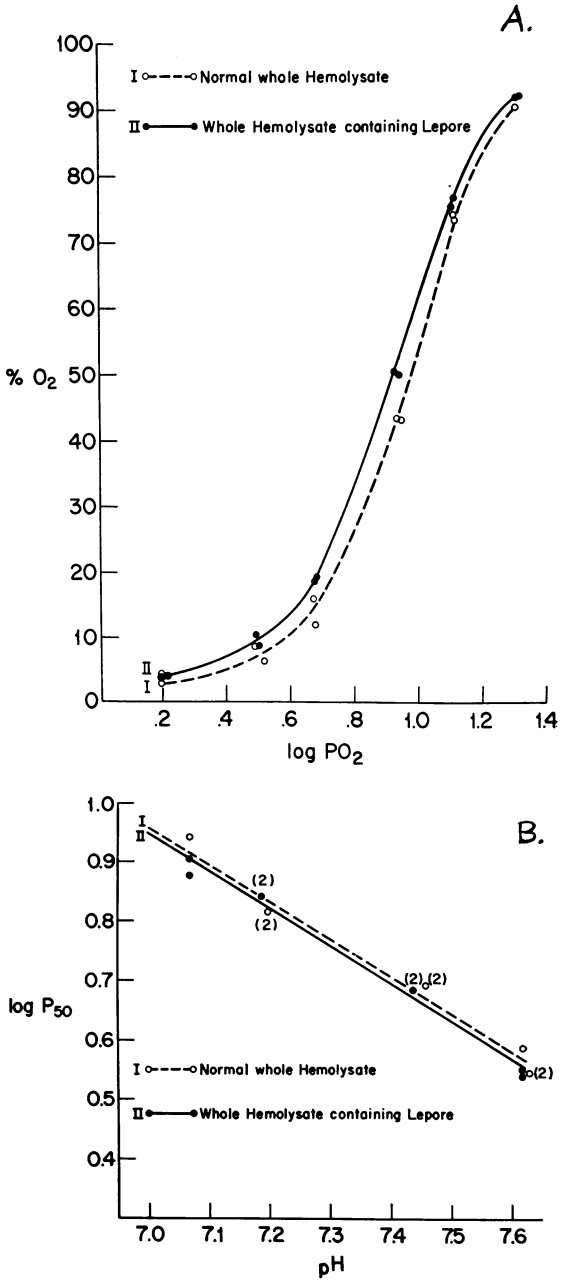

FIG. 1. A. Oxygen dissociation curve. No significant difference in the oxygen affinities of normal whole hemolysate (line I) and whole hemolysate containing Lepore (line II) at pH 7.00, 20° C, was observed. B. The regression of log P_{50} on pH of normal whole hemolysates (line I) and of whole hemolysates containing Lepore hemoglobin (line II) is shown. No significant difference in oxygen affinities was observed. [The symbol (2) represents two identical determinations. The symbol ⊙ represents the coincident values from one determination of the normal whole hemolysate and one determination of the whole hemolysate containing Lepore hemoglobin.] The standard deviation from the regression for the unfractionated hemolysates was 0.0600, and for the hemolysates containing hemoglobin Lepore it was 0.0424. The regression coefficients were homogeneous ($p > 0.5$), and the regression lines did not differ significantly ($0.3 > p > 0.1$) from each other.

Methods

1) Preparation of hemoglobin components. Blood samples were collected in 3.2% sodium citrate from *1)* an 18-year-old male of Italian ancestry, heterozygous for hemoglobin $Lepore_{Einstein}$ (24),[1] for isolation of hemoglobin Lepore (which comprised 12% of the total pigment), *2)* a 58-year-old woman of Italian ancestry with thalassemia minor, in whom hemoglobin A_2 comprised 6% of the hemoglobin, for isolation of hemoglobin A_2, and *3)* normal subjects, for preparation of control hemoglobin A samples. Hemolysates containing hemoglobin in concentrations of 10 g per 100 ml were prepared by the method of Drabkin (25). Samples of hemoglobin Lepore and of hemoglobin A_2 were prepared from the appropriate hemolysate by electrophoresis in 0.05 M Veronal buffer on starch granules or on Pevikon (26) by the method of Kunkel, Ceppellini, Müller-Eberhard, and Wolf (27). Hemolysates from which control hemoglobin A samples were to be prepared were diluted to 1.2 g per 100 ml or to 0.6 g per 100 ml (for comparison with hemoglobin Lepore or A_2, respectively) before electrophoretic separation. Electrophoretic separations of 15 hours duration at 4° C were carried out on solutions of oxyhemoglobin and were completed in less than 24 hours after the blood samples were taken.

At the conclusion of the electrophoretic separation, hemoglobin components were eluted with 0.1 M potassium phosphate buffer, pH 6.5. Several different pH values, ranging from pH 6.80 to 7.65, were achieved by dilution with, or in some cases dialysis against, the appropriate phosphate buffer. Solutions of hemoglobin A_2, and of hemoglobin A that had been diluted to 0.6 g per 100 ml before electrophoresis, were concentrated by ultrafiltration (21) after elution. Values for pH were obtained immediately before studies of oxygen equilibria.

2) Characterization of isolated components. For the present studies, avoidance of methemoglobin formation was considered more important than purity of the isolated components. Consequently no attempts were made to improve the separations by prolonging the duration of electrophoresis or by increasing the current. The average proportion of methemoglobin determined spectrophotometrically on each separated component was 3.5%, with a range of 0.5 to 6.0%. When the studies of oxygen equilibria were completed, the hemoglobin solution was converted to carboxyhemoglobin and concentrated by ultrafiltration, and a second electrophoretic

[1] Preliminary studies of the structure of hemoglobin $Lepore_{Einstein}$ have shown no significant differences from the example of hemoglobin Lepore studied by Baglioni (23).

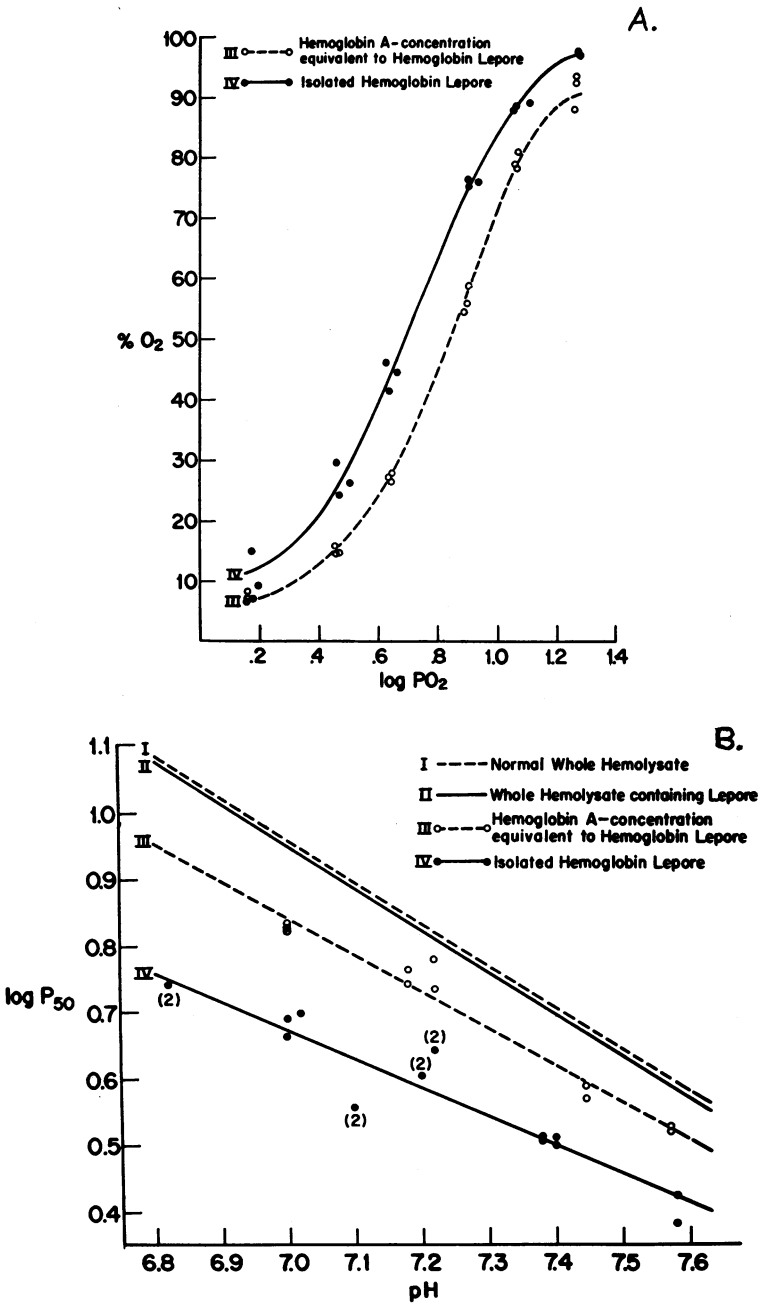

FIG. 2. A. Oxygen dissociation curve. A marked increase in the oxygen affinity of isolated hemoglobin Lepore (line IV) as compared to hemoglobin A at a concentration equivalent to hemoglobin Lepore (line III) at pH 7.00, 20° C, was observed. B. The regression lines for isolated hemoglobin Lepore (line IV) and for hemoglobin A at a concentration equivalent to hemoglobin Lepore (line III) showed a marked increase in oxygen affinity in isolated hemoglobin Lepore. Lines I, II, and III are extended to pH 6.8. The standard deviation of data of isolated hemoglobin A from the regression was 0.0775, and for hemoglobin Lepore it was 0.1539. The regression lines for isolated hemoglobins A and Lepore were markedly different ($0.0005 > p$). Whether regression coefficients were ho-

separation for quantification of components was carried out. When hemoglobin Lepore was studied, the second electrophoretic separation was allowed to proceed for 24 or more hours. It was found that the initial separation of hemoglobin Lepore had yielded solutions containing an average of 70% (range, 62 to 81%) Lepore together with hemoglobin A.

Because large proportions of methemoglobin were sometimes encountered in solutions of hemoglobin A_2 prepared by electrophoresis on starch granules, Pevikon was utilized as the supporting medium for the initial electrophoretic separation of hemoglobin A_2. The separations of hemoglobins A_2 and A on Pevikon were obviously incomplete and 12 to 27% (mean, 24%) contamination of A_2 with A was found on the second (starch granule) electrophoretic separation after completion of studies of oxygen function. Methemoglobin formation was, however, much less on Pevikon than on at least some lots of starch. The data on isolated hemoglobins Lepore and A_2 in the present report were not corrected for the observed contamination with hemoglobin A.

3) Studies of oxygen equilibria. Studies of oxygen equilibria of the isolated components were completed within 30 hours after the blood samples were obtained. (Studies of whole hemolysates were completed within 6 hours.) Determinations of oxygen equilibria were carried out at 20° C on hemoglobin solutions of 0.1 g per 100 ml (0.06 mM heme) concentration, by a modification of the method of Riggs (28) and of Allen, Guthe, and Wyman (29). The oxygen saturation was calculated from measurements of optical density made in a Beckman DU spectrophotometer at 580 and 564 mμ. Adequate deoxygenation of the hemoglobin solutions was indicated by the following ratios for each sample: *1)* the ratio of the optical density of the deoxyhemoglobin at 564 mμ to the optical density at 580 mμ was 1.26 to 1.35 (mean = 1.31) and *2)* the ratio of the optical density of the deoxyhemoglobin at 564 mμ to the optical density of the oxyhemoglobin at 580 mμ was 0.79 to 0.86 (mean = 0.82).

The range of value for n from Hill's equation (30),

$$\frac{y}{1-y} = \left(\frac{P}{P_{50}}\right)^n,$$

in which y represents the fraction of hemoglobin present as oxyhemoglobin, P the oxygen tension, and P_{50} oxygen tension necessary for 50% saturation of the hemoglobin, was 2.3 to 3.1. Determinations that resulted in values for n below 2.3 were discarded (see below).

4) Statistical analyses. The regression of log P_{50} on pH was calculated from the data for each hemoglobin studied by the method of least squares. To test the homogeneity of the regression coefficients (regression coefficients are the slopes of the regression lines) and to compare regression lines of isolated components with hemoglobin A or with unfractionated hemolysates, an analysis of covariance was made (31). If the regression coefficients of the oxygen equilibria of any two hemoglobins were homogeneous, the Bohr effects would be of the same order of magnitude. If the regression coefficients were homogeneous, comparison of the levels of the regression lines would indicate alterations in oxygen affinity. The results of the statistical analyses are summarized in the legends of Figures 1B, 2B, and 3B. (See Table I for the data.)

Results

1) Unfractionated hemolysates. Figures 1A and 1B contain data on unfractionated hemolysates prepared from erythrocytes containing normal hemoglobin (line I) and hemolysates containing 12% hemoglobin Lepore (line II). In Figure 1A, the difference between the oxygen dissociation curves of these two hemolysates at pH 7.00 was not sufficient to be significant. In Figure 1B, the relationships between log P_{50} and different pH values were demonstrated. The regression lines (the slopes of which represent the Bohr effect) for lines I and II appeared about the same. The absence of significant differences in oxygen function between normal unfractionated hemolysates and hemolysates containing 12% hemoglobin Lepore was confirmed on statistical evaluation.

2) Isolated Lepore hemoglobin. In Figures 2A and 2B, data on electrophoretically isolated hemoglobin Lepore (line IV) were compared with normal hemoglobin that had been diluted to approximately the concentration of hemoglobin Lepore before electrophoresis (line III). Figure 2A contains the oxygen dissociation curves of these two hemoglobins at pH 7.00; under these circumstances hemoglobin Lepore appeared to have a greater oxygen affinity than did normal hemoglobin. From Figure 2B, it is clear that hemoglobin Lepore had a greater oxygen affinity at each of several pH values between 6.8 and 7.6.

mogeneous was unclear ($0.10 > p > 0.05$, $p \cong 0.06$), since tests for statistical significance were at borderline values. The regression coefficients for isolated hemoglobin A and normal whole hemolysates were homogeneous ($0.3 > p > 0.1$), although the regression lines were different ($0.0005 > p$). The regression coefficients for isolated hemoglobin Lepore and whole hemolysates containing 12% hemoglobin Lepore were nonhomogeneous ($0.01 > p > 0.005$).

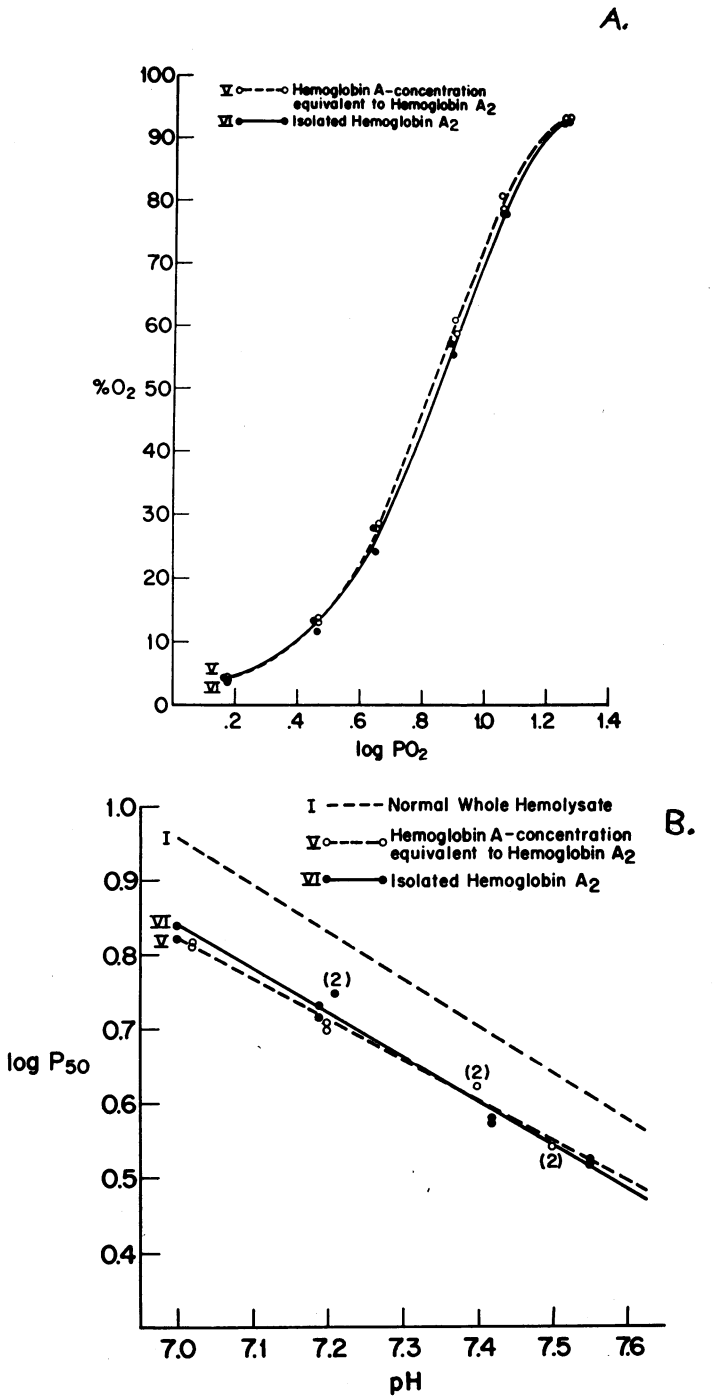

FIG. 3. A. Oxygen dissociation curve. No significant difference in the oxygen affinities of isolated hemoglobin A_2 (line VI) and of hemoglobin A at a concentration equivalent to hemoglobin A_2 (line V) was observed. B. The regression of isolated hemoglobin A_2 (line VI) and of hemoglobin A at a concentration equivalent to hemoglobin A_2 (line V) disclosed no significant differences in oxygen affinities. The standard deviation for the regression of isolated hemoglobin A_2 was 0.0566, and for hemoglobin A at an equivalent con-

The increased oxygen affinity of hemoglobin Lepore was statistically highly significant. Figure 2B also contains the plots for unfractionated hemolysates (lines I and II) from Figure 1B. The difference between hemoglobin A isolated electrophoretically in dilute solution (line III) and normal unfractionated hemolysate (line I) is of interest: the increased oxygen affinity of the isolated normal hemoglobin as compared with whole hemolysate was statistically significant and presumably resulted from alterations in the protein occurring during the isolation procedure. In Figure 2B, the slopes of the regression lines of isolated hemoglobin Lepore and of the isolated normal hemolysates may be different, thus suggesting the possibility of an altered Bohr effect for hemoglobin Lepore. This alteration, however, was of borderline statistical significance.

3) Isolated hemoglobin A_2. Isolated hemoglobin A_2 (line VI) and normal hemoglobin diluted to a concentration equivalent to that of the hemoglobin A_2 before electrophoresis (line V) are compared in Figures 3A and 3B. Figure 3A showed no difference in the oxygen dissociation curves of these two hemoglobins at pH 7.00, and their regression lines demonstrated in Figure 3B appeared the same. These conclusions were supported by statistical analysis.

Discussion

In our studies, identical oxygen dissociation curves of the minor component of normal hemoglobin, hemoglobin A_2, and of the major component hemoglobin A were observed. Rossi-Fanelli and co-workers (9) obtained similar data for hemoglobins A_2 and A, whereas others (10, 11) have found hemoglobin A_2 to have a higher oxygen affinity than hemoglobin A. No significant difference in the Bohr effect of hemoglobin A_2 as compared to hemoglobin A was observed in these or previous studies. The differing conclusions concerning oxygen affinity of hemoglobin A_2 may have resulted from differences in techniques of isolating the components. For the present studies and for those of Rossi-Fanelli and co-workers (9), zone electrophoresis was employed; our studies of oxygen equilibrium were completed within 30 hours after the blood was shed. Huisman and co-workers (10, 11), however, isolated hemoglobin A_2 by DEAE cellulose chromatography, and approximately 72 hours elapsed before determinations of oxygen equilibria. In the present studies, a higher oxygen affinity of isolated hemoglobin A was noted when hemoglobin A was diluted before rather than after the electrophoretic separation. Therefore, control hemoglobin A was diluted to the same concentration as hemoglobin A_2 or hemoglobin Lepore before the electrophoretic separations.

The interaction constant, n, defined by Hill's equation, is approximately 3 for normal human hemoglobin. The oxygen dissociation curves of hemoglobin A_2 and hemoglobin Lepore exhibited no evidence of decreased heme-heme interaction when compared to similar data on isolated hemoglobin A. Values for n, however, were about 2.6 for each of the three isolated components: A, A_2, and Lepore. These changes in the interaction constant presumably resulted from changes in the protein during the isolation procedure. Similar values for n were obtained by Rossi-Fanelli and co-workers (9). (Studies with a value for n of 2.3 or lower probably represented excessive denaturation and were discarded in the present study.) The n values obtained by Huisman and co-workers (11) in studies of isolated hemoglobin A_2 ranged between 1.4 and 2.0.

The increased oxygen affinity of isolated hemoglobin Lepore demonstrated in these studies resembled that observed by Huisman and co-workers (18). The expected and observed effect of this increase in oxygen affinity of hemoglobin Lepore on the unfractionated hemolysate was so

centration it was 0.0360. The regression coefficients for these two hemoglobins were homogeneous ($0.5 > p > 0.3$), and the regression lines were not significantly different ($0.5 > p > 0.3$). The regression coefficients for isolated hemoglobin A_2 and unfractionated normal hemolysates were homogeneous ($p > 0.5$), although the regression lines differed ($0.0005 > p$). The regression coefficients of hemoglobin A at a concentration equivalent of hemoglobin A_2 and unfractionated normal hemolysates were homogeneous ($0.3 > p > 0.1$); these regression lines were also different ($0.0005 > p$).

slight as to be within experimental error. In addition, the data were compatible with a small difference in the magnitude of the Bohr effect of isolated hemoglobin Lepore when compared with hemoglobin A at an equivalent concentration. Since denaturation in general increases the oxygen affinities of hemoglobins, it is possible that the increased affinity of hemoglobin Lepore is due to an increased amount of denaturation. However, since the values for n were about the same for hemoglobin Lepore and hemoglobin A, there is no evidence of any major difference in the amount of denaturation. Furthermore, the concentrations of methemoglobin in the two hemoglobins were also approximately the same. Since Lepore amounts to only 12% of the total hemoglobin, comparison of oxygen equilibria of the isolated component with those of hemolysates or of erythrocytes containing hemoglobin Lepore would not provide crucial evidence concerning denaturation.

Benesch, Ranney, Benesch, and Smith (21) pointed out that interactions between α and β chains were apparently essential for three important aspects of function of hemoglobin A: the Bohr effect, the sigmoid shape of the oxygen dissociation curve (n), and the oxygen affinity. From observations on dissociation and hybridization, it may be concluded that the interchain relationships between the α and γ chains of hemoglobin F (32, 33) and between the α and δ chains of hemoglobin A_2 (34) are different from the interchain relationships of hemoglobin A ($\alpha_2^A \beta_2^A$). These differences in interchain bonds do not, however, result in differences in the parameters of oxygen function between hemoglobins F, A_2, and A. In hemoglobin Lepore, two normal α chains combined with two chains that have δ-like N-terminal and β-like C-terminal portions, an altered oxygen affinity was observed. The altered oxygen affinity is apparently related to the presence of the unusual non-alpha chains and suggests that the interchain interactions of hemoglobin Lepore differ from those of either of the normal hemoglobins A_2 or A.

Summary

The oxygen equilibria of hemoglobins A, Lepore, and A_2 isolated by zone electrophoresis were studied. The oxygen equilibrium of hemoglobin A_2 did not differ significantly from that of hemoglobin A subjected to the same experimental conditions. A significant increase in oxygen affinity was demonstrated in isolated hemoglobin Lepore as compared with isolated hemoglobin A. The expected and observed effect of this increased affinity on unfractionated hemoglobin from a Lepore heterozygote was so slight as to be within experimental error. Isolated hemoglobins A, A_2, and Lepore appeared to have the same heme-heme interaction and each exhibited a Bohr effect, although the Bohr effect of hemoglobin Lepore might have differed from that of hemoglobin A. The increased oxygen affinity of hemoglobin Lepore may be an effect of altered interchain relationships resulting from the presence of the unusual non-alpha chains in the tetramer.

Acknowledgments

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