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Hysteresis-Like Behavior of Oxygen Association–Dissociation Equilibrium Curves of Sickle Cells Determined by a New Method (39659)

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It is generally accepted that sickle cell anemia is the consequence of polymerization of mutant hemoglobin (HbS) upon deoxygenation. The mechanism of intracellular polymerization, however, is complicated by the presence of hemoglobin variants and the manner in which polymerization is brought about.

Recently, Cameron (1) observed that the oxygen affinity of sickle red blood cells (S-rbc) was greater when the equilibrium curve was determined during the process of deoxygenation by one method (2) as compared to that determined during the process of reoxygenation by a still different method (3). Rossi-Bernardi *et al.* (4) claim that the oxygenation equilibrium curves of homozygous S-rbc hemolysates after initial deoxygenation are similar to those obtained for intact normal red blood cells (rbc), whereas those of intact S-rbc show lowered oxygen affinity. Whether a change in concentration of intracellular constituents such as 2,3-diphosphoglycerate, HbF content, intracellular polymerization of HbS, pH, or other factors, is responsible for the above results needs to be shown by direct comparison of the deoxygenation and reoxygenation processes for the same S-rbc sample.

Using a newly developed instrument, the authors of the present paper have made this comparison and have determined that the initial rate of deoxygenation of S-rbc has a significant effect on the reoxygenation process.

Instrumentation. Various methods exist for the continuous determination of either oxygen association or oxygen dissociation equilibria of rbc (2–5). However, none of these methods allows the determination of both using the same sample. The instrument described herein uses less than a few drops of blood to obtain continuous curves for both oxygenation and deoxygenation equilibria starting from any desired level of oxygen saturation. Less than 5 min is required for each direction of equilibrium determination.

A simplified diagramatic sketch of this instrument is shown in Fig. 1. Whole blood is suspended as a thin liquid film on EMI micromesh in the thermostated chamber (Fig. 2). The suspended rbc within this reenforced thin film quickly establish gaseous equilibrium with the surrounding gas. Two types of gases, A and B, are mixed, saturated with water vapor, and then pumped at a near-constant net flow rate from the left of the thermostated chamber. The mixing ratio of the gases changes continually. However, the rate of change of mixing is maintained so that rbc suspended in the thin liquid film are always in equilibrium with the gas, and the rate does not exceed the response time of the Clark-type oxygen electrode. The electrode, mounted close to the rbc suspension, monitors the partial pressure of oxygen. The depolarizing current from the electrode is amplified and fed into the X axis of an X–Y recorder. Two independent wavelengths, λ_1 and λ_2, are obtained from an Aminco–Chance dual wavelength spectrophotometer. Normally, λ_1 is set at 431 nm (the Soret band of deoxygenated rbc), and λ_2 is set at 396.5 nm (the isobestic point). Spectral changes observed with the end-on photomultiplier are amplified and fed to the Y axis of the recorder.

The behavior of this instrument requires the uniformity and strength of the thin layer film. EMI nickel micromesh No. 100, 5-μm thick, provides a reproducible, sufficiently thin, liquid film, while restricting the translational motion of rbc. Thus, the number of rbc "seen" by the photomultiplier is constant, and unwanted absorbance fluctuations are reduced. The thin film of blood formed on the micromesh support can with-

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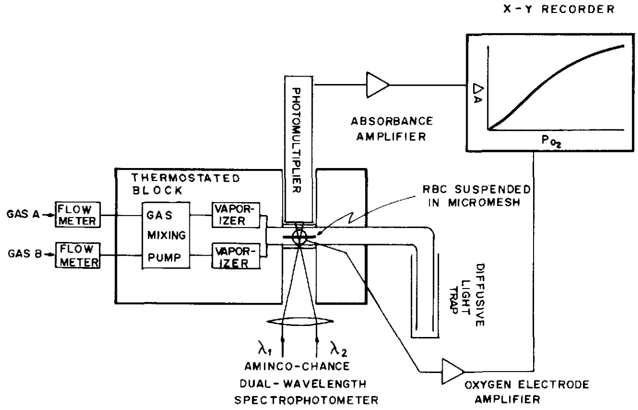

FIG. 1. A simplified diagramatic sketch of the instrument.

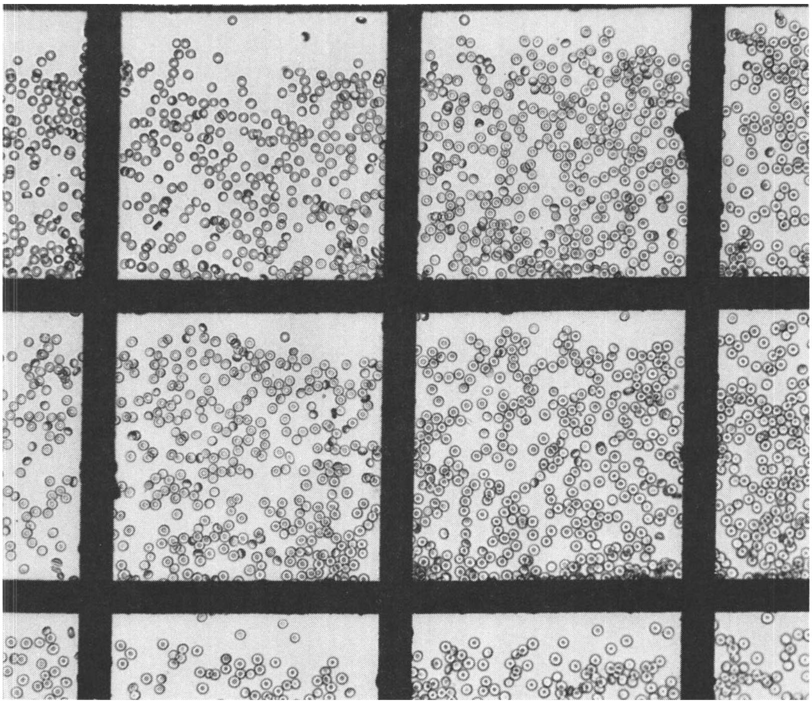

FIG. 2. Microscopic photograph of red blood cells suspended in EMI micromesh No. 100.

stand up to 20 min of constant purging by gas saturated with water vapor and flowing at the rate of 200 ml/min or more. Microscopic observation of the sample after 20 min of purging with gas did not reveal significant structural change of the rbc.

In addition to the thickness of the blood film, the response time of the Clark-type oxygen electrode is also a limiting factor for the rate of change of the gas mixing. The latter is less than 3 sec for a 90% response. A thin liquid junction lying between the electrode and the membrane is formed by pressing a drop of 3 *M* KCl hard against the polished flat electrode surface with a Teflon membrane 0.5 mil thick (DuPont or equivalent). The most rapid experimentally determined gas exchange rate with a reversible trace is 2 min in each direction. The oxygen electrode is calibrated with N_2 and 25% O_2 gases.

Test of instrument with normal blood.

Blood samples were collected from healthy subjects into heparinized Vacutainer tubes. After addition of small amounts (normally 10% by volume) of 0.9% NaCl, samples were used immediately. All experiments were carried out at 29 ± 0.5°C.

(*a*) Test for reversibility of the oxygen association-dissociation equilibrium curve: One drop of the sample was placed on the micromesh, and excess blood was removed. The mesh was placed into the reaction chamber which was preflushed with a gas mixture composed of 5% CO_2, 25% O_2, and 70% N_2 (henceforth referred to as 5% CO_2-O_2 gas). By activating the gas mixing pump, a gas mixture composed of 5% CO_2 and 95% N_2 (henceforth referred to as 5% CO_2-N_2 gas) was gradually introduced while the pumping rate of 5% CO_2-O_2 gas was reduced. In general, complete exchange of the two gases was accomplished within approximately 5 min. The net flow of the gas mixtures was maintained at ca. 200 ml/min throughout the experiment. After completion of the gas exchange, i.e., deoxygenation, the process was reversed by reintroducing 5% CO_2-O_2 gas. Several more experiments were carried out with the same sample and P_{50} values were obtained.

(b) The Bohr effect: Equilibrium curves were obtained using gas mixtures containing 2.5, 5.0, and 10.0% CO_2.

Typical oxygen-rbc equilibrium curves obtained during the process of deoxygenation (the arrow going from right to left) and that of reoxygenation (the arrow going from left to right) are shown in Fig. 3. The X axis of the recorder was calibrated with gases containing 25 and 0% O_2 and was converted to read Po_2 in mmHg after correcting for vapor pressure at that temperature and atmospheric pressure. The Y axis of the recorder monitored oxygen saturation of rbc's, assuming the rbc's were fully saturated in gas containing 25% O_2 and deoxygenated in gas containing 0% O_2. As can be seen in Fig. 3, the equilibrium curves obtained during the process of deoxygenation and reoxygenation are nearly identical, confirming that equilibrium was maintained at all times. The half-saturation point, P_{50}, thus obtained, is 19.5 mmHg and is close to that tabulated elsewhere (6) at this temperature and CO_2 content.

Figure 4 shows the saturation curves obtained during the process of deoxygenation using a gas mixture containing 2.5, 5.0, or 10% CO_2. Although not shown, the reversibility of the curves (for both deoxygenation and reoxygenation processes) under present conditions has been confirmed. The fact that all three curves do not have an identical total absorbance change is primarily due to the differences in the populations of rbc's seen by the photomultiplier tube. The average P_{50} for gas mixtures containing 2.5, 5.0, and 10.0% CO_2 are 16.8, 19.3, and 24.5 mmHg, respectively, and are summarized in Table I. After correcting for temperature and Pco_2, these values are similar to those reported elsewhere (6).

Experiments with S-rbc. S-rbc were obtained from a homozygous sickle cell anemia patient (HbS 87.0% and HbF 10.5%), and the sample was used within 4 hr after collection of the blood in heparinized Vacutainers (equivalent to two drops of sodium heparin in 10 ml of blood). Homozygous sickle cell blood from two other individuals was also tested. However, due to the varia-

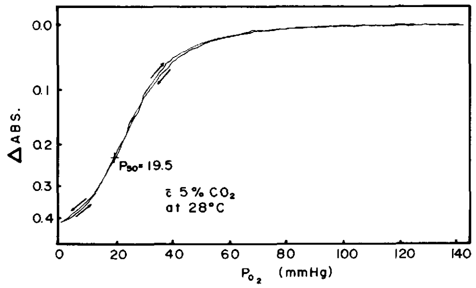

FIG. 3. Oxygen association–dissociation equilibrium curves of whole blood at 28° in 5.0% CO_2. The direction of the arrows indicates the change of partial pressure of oxygen.

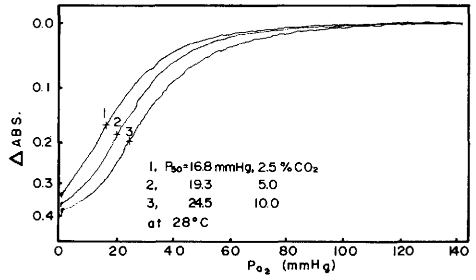

FIG. 4. The oxygen dissociation equilibrium curves of whole blood in 2.5, 5.0, and 10.0% CO_2 at 28°.

TABLE I. HALF OXYGEN SATURATION PRESSURE OF WHOLE BLOOD AT VARIOUS CO_2 LEVELS AT 28°C.

CO_2 (%)	P_{CO_2} (mmHg)	$t_{1/2}$ (mmHg)[a]	P_{50} (mmHg)[b]
2.5	17.9	13.6	16.8[c]
5.0	35.8	16.7	19.3 ± 1.2[d]
10.0	71.6	21.0	24.5[c]

[a] Ref. (6).
[b] This experiment.
[c] Average of three.
[d] From six runs.

bility of hemoglobin composition in each individual blood sample, no quantitative comparison among the subjects will be attempted at this time. The individuals tested had not been on chronic drug therapy or had any medical treatment during the last 24 hr. The tracing of the curve in the direction of either deoxygenation or reoxygenation took approximately 10 min. Although not shown, temporary maintenance at any particular partial pressure of oxygen did not show a significant change in absorbance, indicating that the S-rbc and the environmental gases were in equilibrium.

Results. Typical oxygen association-dissociation equilibrium curves of S-rbc are shown in Fig. 5. The curves obtained during deoxygenation are shown in continuous lines, and those obtained during reoxygenation are shown in broken lines. The heavy lines represent experiments in 10.0% CO_2 and the thin lines represent those in 5% CO_2. As can be seen in each CO_2 system, the curve obtained at the time of reoxygenation is shifted to the right of those obtained during deoxygenation, thereby displaying hysteresis-like phenomena. Similar experimental results for two other homozygous patients (F. S. and E. S.) are shown in Fig. 6. It can be seen that, in both samples, hysteresis-like phenomena persist.

After replotting the Fig. 5 results as Hill plots (Fig. 7), the P_{50} and Hill constant, n (at P_{50}), of each curve are obtained, and the results are summarized in Table II. The P_{50} of blood depends upon 2,3-diphosphoglycerate, pH, Pco_2, temperature, and the content of hemoglobin variants (11). Accordingly, P_{50} values reported here are not necessarily representative of S-rbc. In addition to the hysteresis-like phenomena described, it can be seen that the Hill constants at P_{50} for both CO_2 systems are less at the time of reoxygenation than are those of deoxygenation. The change in the constant was greater for the 10% CO_2 system. As has been observed by others (7, 8), the Hill constant at P_{50} is relatively small for the equilibrium curve at the time of reoxygenation, but increases considerably as the degree of oxygen saturation increases. This apparent increase in cooperativity in the higher region of oxygenation may be related to the depolymerization of HbS.

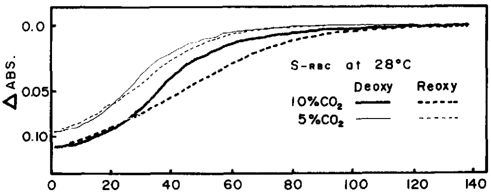

FIG. 5. The oxygen association-dissociation equilibrium of sickle cells at 28°. The curves obtained during deoxygenation are shown in continuous lines, and those obtained during reoxygenation are shown in broken lines. The heavy lines represent experiments in 10.0% CO_2, and thin lines are for 5.0% CO_2.

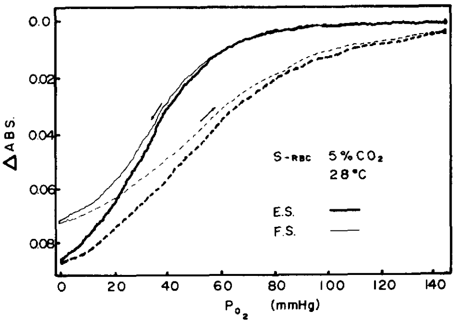

FIG. 6. The oxygen association-dissociation equilibrium curves of sickle cells from two other homozygous patients at 28° in 5% CO_2.

Discussion. It has been observed that the oxygen dissociation equilibrium curve of dilute HbS in solution resembles that of normal hemoglobin (HbA) in solution (9, 10). The oxygen dissociation equilibrium curve of S-rbc, on the other hand, is shifted to the right of that of normal rbc. While neither phenomenon has been adequately explained, the intracellular level of 2,3-DPG, the hemoglobin concentration, the level of HbF, the pH, the degree of gelation, and the properties of cellular membranes have been considered responsible for the shift in the curve of S-rbc. Technical difficulties,

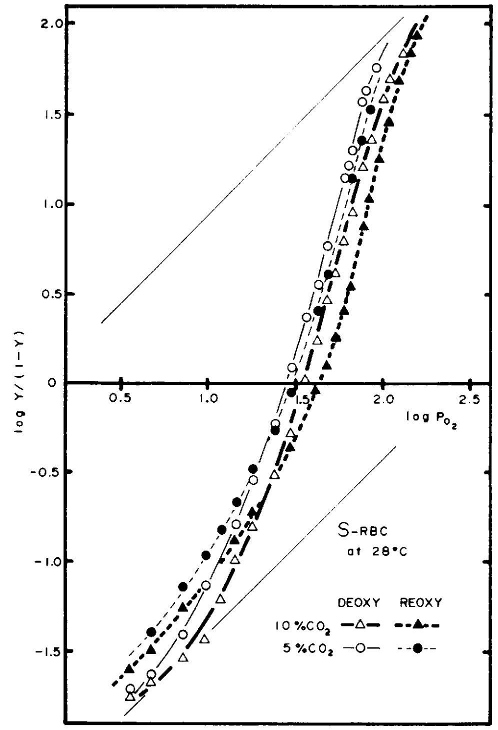

FIG. 7. Hill plot of Fig. 5.

TABLE II. OXYGEN AFFINITY (P_{50}) AND HILL'S CONSTANT (n) OF HOMOZYGOUS SICKLE CELLS DETERMINED AT 28°C.[a]

CO_2 (%)	P_{50} (mmHg)		n (at P_{50})	
	Deoxy	Reoxy	Deoxy	Reoxy
5.0	28.2	31.3	3.0	2.7
10.0	31.8	43.7	3.25	2.5

[a] Average of three runs.

however, prevented the investigators from comparing the oxygen equilibrium curves at the time of deoxygenation and reoxygenation using the same S-rbc. An experiment performed within the period that causes little or no intracellular chemical change will provide evidence for the difference observed between the two equilibrium curves being attributable to a change in physical status. To this end it is important to note the change of physical status observed in HbS solution.

According to Eaton *et al.* (12), the critical temperature of the gelation of deoxygenated HbS in solution is dependent upon the rate of temperature change. Specifically, the critical temperature is lower if the sample is warmed slowly. This observation implies that the thermal motion of deoxygenated HbS becomes the rate-limiting step when the solution temperature is rapidly changed. This thermal motion of HbS will directly affect the number and size of polymer crystals serving as nuclei of crystallization. On the other hand, upon deoxygenation of S-rbc, there occurs a conformational transition of HbS from R to T state, and gelation (or polymerization) will be initiated. It is then possible that the rate of deoxgyenation can also affect the number and size of seed nuclei formed intracellularly. If sickling is caused by a rapid deoxygenation process, the polymers formed are less ordered, or inhomogeneous. Josephs *et al.* (13) also observed at least two types of helical fibers. Curve 1 in Fig. 8 was obtained at the time of reoxygenation of S-rbc which had previously been quickly deoxygenated by insertion of the sample into the deoxygenation chamber. Immediately after completion of reoxygenation, curve 2 was obtained by slowly deoxygenating the same sample in the usual manner. Curve 3 was obtained by slowly reoxygenating the sample a second time. Curves 1 and 2 are similar in that they are both sigmoidal and have approximately the same oxygen affinity, whereas only curve 3 shows an apparent reduction in the sigmoidal shape and a decrease in oxygen affinity.

Based on these observations we suggest that: During deoxygenation, sickle cell hemoglobin interacts as independent molecules until a certain degree of deoxygenation, at

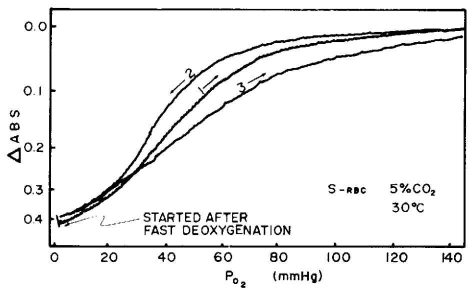

FIG. 8. The first oxygen association equilibrium curve (1) was obtained by placing the sample directly into the previously deoxygenated chamber, thereby deoxygenating the sample quickly. After completely reoxygenating the sample, it was slowly deoxygenated (curve 2) and reoxygenated slowly (curve 3). Reduced sigmoidness is observed only in curve 3.

which point the nucleation of the molecule takes place. The molecule will be further polymerized as the deoxygenation continues to completion. When reoxygenation is initiated, the polymeric form, for which oxygen affinity is lower than for the nonpolymeric form, will be maintained despite a considerably higher partial pressure of oxygen, thereby presenting an apparent shift of the equilibrium curve to the right. On the other hand, polymers formed during rapid deoxygenation accomplish depolymerization at a lower partial pressure of oxygen, hence, only a small shift of the equilibrium curve is observed. Although these conclusions are only qualitative and need further study, it can be assumed that the hysteresis-like behavior of the oxygen association–dissociation curves at the temperatures studied results from the formation of specific intracellular polymers during the time of deoxygenation and that variation in polymer structure occurs in varying rates of deoxygenation, causing variation in the reoxygenation curve.

Summary. An instrument to determine the oxygen association–dissociation equilibrium of whole blood has been developed. Only one drop of blood sample is required to trace automatically the oxygen–red blood cell equilibrium curve in the direction of deoxygenation and reoxygenation within a total of less than 10 min. The whole blood is suspended in the form of a thin liquid film reenforced by a micromesh. The partial pressure of oxygen is changed continuously with a gas-mixing pump while simultaneously being recorded by polarographic oxygen electrodes. The spectral change of red blood cells is recorded by a dual-beam spectrophotometer.

Using this instrument it is shown that the oxygen equilibrium curve of sickle cells obtained during deoxygenation is shifted to a higher oxygen affinity as compared to those obtained during reoxygenation. This hysteresis-like behavior of equilibrium curves is also reflected in the Hill constant, n, which is greater for deoxygenation than for reoxygenation. On the other hand, if deoxygenation is completed rapidly, the sigmoidness of the reoxygenation equilibrium curve and its oxygen affinity are greater than that obtained after slow deoxygenation. Hysteresis-like behavior is the postulated consequence of intracellular polymerization of sickle hemoglobin.

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