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Reconstitution of native human hemoglobin from separated globin chains and alloplex intermediates

(*in vitro* assembly of protein/oxygen binding curves/hemoglobin subunits)

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ABSTRACT **A complete experimental format is given for the reconstitution of human hemoglobin from the separated heme-free α- and β-globin chains (α°, β°) and hemin, by two alternative routes. Based on their oxygen binding properties, the reaction of the ferri-forms with reducing agent, and the response of the oxygen binding curves to pH variation and to the addition of the allosteric effector 2,3-diphosphoglycerate, the molecules are native. One reconstitution route uses direct addition of hemin to the separated globin chains with production of the separated subunits, which can then be recombined and reduced. This procedure occasions losses by precipitation in the heme-addition step except at high dilutions, and the yields are low. In the second pathway, either globin chain is mixed with the complementary untreated subunit to form the half-filled (with heme) intermediates, which combine stoichiometrically with hemin. No precipitation accompanies these reactions. For α-globin, the yield is about 50% because of incomplete combination with the heme-containing β chain. For β-globin, the yield is better than 70%. It is suggested that experiments intended to test either globin chain should use the second route in preparation for structural or functional comparisons with native hemoglobin.**

In two previous papers (1, 2), we described the preparation and properties of isolated heme-free human globin chains (α°, β°) and of two kinds of reassembly intermediates in which both kinds of chains are present but only half of the heme sites are filled (HFα and HFβ molecules). The first paper demonstrated that the separated globin chains near neutral pH have different conformations, α° being substantially more disordered than β°, in contrast to the separated heme-containing subunits, which are folded similarly, and in contrast also to the heme-free chains when bound together in dimeric apohemoglobin ($\alpha^\circ\beta^\circ$). Moreover, it was shown that, once separated, the globin chains do not readily recombine at neutral pH and accessible temperatures to form apohemoglobin. The second paper demonstrated that if either chain contains a heme, then the complementary chains do specifically recombine. During such a recombination, the disordered α-globin is refolded to the conformation it possesses in apohemoglobin. The coupled binding and refolding was termed an alloplex interaction†, to emphasize the extensive refolding induced by a neighboring subunit.

In future papers on this subject, the kinetics and mechanism of the alloplex interaction and the equilibrium of the HFα and HFβ intermediates will be reported. The present paper shows that human hemoglobin can be reconstituted from separated globin chains and hemin by two different routes and that the reconstituted hemoglobin is similar or identical to native hemoglobin with respect to cooperativity in oxygen binding, the Bohr effect, and response to the allosteric effector 2,3-diphosphoglycerate (P_2-glycerate).

In the course of our work on this problem, we found it necessary to modify several of our previously published experimental procedures, ranging from the separation and regeneration of hemoglobin subunits to the preparation of the globin chains. The modifications were introduced to improve yield and to meet the increasingly rigorous demands placed on tests of functionality of the finally reconstituted hemoglobins. In particular, the published procedures for preparation of β-globin chains led to β-subunits after reconstitution that were, by physico-chemical tests of structure, virtually identical to native subunits, although minor differences were noted and discussed. However, when these subunits were incorporated into reconstituted hemoglobin, the oxygen dissociation curves were noticeably deviant from those of native Hb A (e.g., they did not respond precisely to addition of P_2-glycerate) whereas spectral tests and features of chemical reactivity were not discernibly different.

To avoid confusion about procedures finally adopted in all steps of the dissociation, removal of prosthetic group, and reconstitution in any of several ways, we present in this paper the entire experimental format. Referred to globin chains as starting material, the yields are now about 50% for α-globin and better than 70% for β-globin, at total protein concentrations of several percent, when the reconstitution is through the HF intermediates.

MATERIALS AND METHODS

Hemin, *p*-mercuribenzoate (PMB), P_2-glycerate, phenazine methosulfate, NADH, and EDTA were highest purity grade, purchased from Sigma Chemical Co. All buffer solutions used in the experiments reported contained 1 mM EDTA, and all experiments were carried out at 4° unless stated otherwise.

Preparation of Hemoglobin. Human hemoglobin was prepared as previously described (1, 2) at concentrations of 10–14%.

Preparation of Separated Subunits. The experimental conditions described here are for working with 10–14 g of hemoglobin in a volume of up to 100 ml. Hemoglobin was disso-

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† In a previous paper (2), we identified $\alpha^\circ\beta^h$ as predominantly tetrameric and $\alpha^h\beta^\circ$ as dimeric, primarily on the basis of elution patterns obtained on Bio-Gel columns. Our present results still indicate that $\alpha^h\beta^\circ$ is dimeric, but $\alpha^\circ\beta^h$ behaves largely as a dimer on molecular sieve columns and in the ultracentrifuge. Whether this is due to the better resolving power of the Ultrogel AcA 54 columns or to the modifications in preparation and purification of α° is not definitely established. A full description of the molecular weight distributions of half-filled molecules under a variety of conditions will be published elsewhere (Y. K. Yip, J. Luchins, and S. Beychok, manuscript in preparation).

Abbreviations: ° attached to an α or β indicates that the chain has no heme attached; ^{h} indicates that the chain has heme bound; HFα, heme is attached to the α-chain only; HFβ, heme is attached to the β-chain only; $_R$ and $_N$, reconstituted and native chains, respectively; P_2-glycerate, 2,3-diphosphoglycerate; Bis-Tris, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; PMB, *p*-mercuribenzoate.

ciated into its subunits by reaction with PMB, according to Bucci and Fronticelli (3). PMB, 100 mg/g of hemoglobin, was first dissolved in a minimum volume of 0.1 M KOH, and 1 M acetic acid was added dropwise until a very slight precipitate persisted. The hemoglobin solution was adjusted to contain 0.2 M NaCl and brought to pH 6.0 by addition of 1 M KH_2PO_4. The PMB and hemoglobin solutions were then mixed thoroughly and adjusted to pH 6.0 by adding 1 M acetic acid. The PMB-Hb solution was allowed to stand at 4° for 12–14 hr.

The procedure for the separation of α- and β-PMB subunits is based on that of Geraci *et al.* (4), with modifications. For the preparation of α-subunits, one half of the PMB-Hb solution was equilibrated with 50 mM Tris·HCl buffer, pH 8.4, by filtration through a Bio-Gel P-2 column (5 × 40 cm). The equilibrated solution was then applied to a DEAE-cellulose column (2.5 × 30 cm) equilibrated with the above buffer. The α-PMB subunits were eluted also with the same buffer.

For the preparation of β-subunits the remaining half of the PMB-Hb solution was brought to pH 6.4 by filtration through a Bio-Gel P-2 column (5 × 40 cm) equilibrated with 20 mM potassium phosphate, pH 6.4. It was then applied to a similarly equilibrated CM-cellulose column (2.5 × 40 cm), and the β-PMB subunits were eluted with the same buffer.

Both the α-PMB and β-PMB solutions, as well as all other heme-containing proteins in these experiments, were saturated with carbon monoxide and dialyzed against 0.1 M potassium phosphate, pH 7.4 at 4°, whenever overnight storage became necessary.

The separated subunits collected from the above procedures were usually in volumes between 150 and 250 ml, and each of these subunits was found, occasionally, to contain Hb and the other subunit. The following procedures were carried out to concentrate as well as to purify the separated subunits.

The α-PMB solution was dialyzed against 20 mM potassium phosphate at pH 6.4, and the β-PMB solution was dialyzed against 50 mM Tris·HCl at pH 8.4. In both cases, overnight dialysis was required with two changes of a total of 8 liters of each buffer. The α-PMB solution was loaded on a CM-cellulose column (2.5 × 12 cm) and the β-PMB solution was loaded on a DEAE-cellulose column (2.5 × 12 cm), each equilibrated with the same buffer used for dialysis. The desired subunits were retained on each of the cellulose columns, and any contaminating subunit was washed out by the dialysis buffers. Hemoglobin, in turn, was removed by 20 mM, pH 7.0, potassium buffer from the CM-cellulose column, and by 20 mM, pH 7.5, potassium phosphate buffer from the DEAE-cellulose column. The α-PMB subunit was finally eluted with 0.1 M potassium phosphate, pH 7.5, and the β-PMB with 0.1 M potassium phosphate, pH 7.0, each in a volume of 50–70 ml.

Regeneration of α-SH Subunit. Up to 70 ml of a 2–4% α-PMB solution was applied to a Bio-Gel P-2 column (5 × 45 cm) that was equilibrated with 0.1 M potassium phosphate, pH 7.4, containing 25 mM 2-mercaptoethanol. The α-subunit was eluted with the same buffer, and the flow rate was adjusted so that the α-SH subunit would be eluted completely from the column in 30–45 min. The solution of α-SH subunit was passed immediately through a second Bio-Gel P-2 column (5 × 85 cm) equilibrated with 0.1 M potassium phosphate, pH 7.4, for removal of excess mercaptoethanol. The exposure of α-subunit to a higher concentration of mercaptoethanol or to the same concentration for longer periods caused extensive oxidation.

Regeneration of β-SH Subunit. β-PMB subunits, at a concentration of 2–4% in a volume up to 70 ml, were incubated in 0.1 M potassium phosphate buffer at pH 7.4, 50 mM mercaptoethanol for 1 hr at 4°. The PMB was removed on a Bio-Gel P-2 column (5 × 45 cm) equilibrated with the same buffer. The β-SH subunits were then freed of excess mercaptoethanol by filtration through another Bio-Gel P-2 column (5 × 85 cm) equilibrated with 0.1 M potassium phosphate, pH 7.4.

These regeneration procedures produce one SH group per α-subunit and two SH groups per β-subunit with no appreciable oxidation of the heme and no loss of the starting materials.

Preparation of Separated Heme-Free Globin Chains. For a successful preparation of the separated globin chains, it is important to render the heme protein solutions as nearly salt-free as possible prior to the removal of heme in acid/acetone.

In the case of the β-SH subunit this is readily accomplished by filtration through a Bio-Gel P-2 column (5 × 85 cm) equilibrated with deionized water. The solution of α-SH subunit, however, can only be partially desalted through a Bio-Gel P-2 column equilibrated with 5 mM potassium phosphate, pH 7.4, since an appreciable amount of the α-SH subunits is retained by the polyacrylamide gel when it is equilibrated with deionized water.

Heme protein solutions at a concentration between 1 and 3% were added dropwise at −16° with vigorous stirring to an extraction mixture consisting of 3 ml of 2 M HCl in 1 liter of acetone (1:20, vol/vol). Stirring was continued for 15 min after the addition was completed. The globin precipitates were washed three times with acetone at −16° and centrifuged at that temperature at 12,000 × *g* for 5 min. Both globin precipitates were dissolved in 20–30 ml of 20 mM K_2HPO_4, as required to bring the initial pH of the globin solutions to a value between 4.0 and 4.5. The α-globin solution was then dialyzed exhaustively against 20 mM potassium phosphate, pH 6.7, with four changes of 2 liters each, for 48 hr; and the β-globin solution was similarly dialyzed against 20 mM potassium phosphate, pH 5.7. The α-globin solution was passed through a 2.5 × 85 cm molecular sieve column (Ultrogel AcA 54, LKB Instruments, Inc.) equilibrated with 20 mM potassium phosphate, pH 6.7, and the β-globin solution was passed through a similar column equilibrated with 20 mM potassium phosphate, pH 5.7. Fractions measuring 5 ml were collected at a flow rate of 30 ml/hr, and the elution profile was scanned at 280 nm. The major α-globin peak is a high-molecular-weight aggregate (1) and is excluded by the gel; the major β-globin fraction is a dimer. Only these fractions were used in subsequent reconstitutions.

Reconstitution of Subunits and Reassembly of Hemoglobin. Hemin, 10 mg, was dissolved initially in 1 ml of 0.1 M NaOH, then diluted with water to a final volume of 25 ml. Concentration was determined spectrophotometrically using the value of 5.0×10^4 as the molar extinction coefficient (5). The hemin solution prepared in this manner caused only negligible change in the pH of the α- and β-globin solutions upon its addition. Globin concentrations were determined spectrophotometrically using molar extinction coefficients of 1.0×10^4 and 1.54×10^4 for α- and β-globins, respectively (1). Hemin was always added in 1.2-fold molar excess over the globin. The reconstituted α- and β-subunits were dialyzed against 0.1 M potassium phosphate, pH 7.4, before use for further experiments. Excess heme was removed by passing through a Bio-Gel P-2 column equilibrated with the appropriate buffer. Concentrations of the reconstituted subunits were determined spectrophotometrically as the cyanmet derivatives (1, 2). Reassembly of hemoglobin was carried out by (*i*) mixing equimolar quantities of the reconstituted ($_R$) subunits, yielding $(\alpha_R\beta_R)_2$ or (*ii*) mixing each of the reconstituted subunits with the complementary native ($_N$) ones, producing $(\alpha_R\beta_N)_2$ and $(\alpha_N\beta_R)_2$, and then adding stoichiometric amounts of hemin to

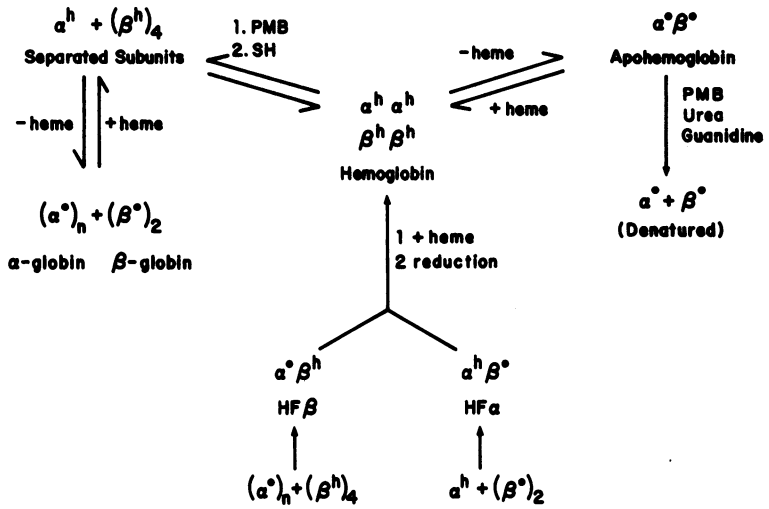

FIG. 1. Separation and reassembly scheme of human hemoglobin. See *Materials and Methods* for details of the experimental procedures. Subscript n for α° indicates that the α-globin is an aggregate having a molecular weight greater than 1.5×10^5 and a $s_{20,w}$ value of 6.6 S (1).

the half-filled (HF) intermediates (Fig. 1). The half-filled molecules were prepared as described by Waks *et al.* (2). It was necessary, however, to pass the reaction mixtures through a molecular sieve column (Ultrogel AcA 54) to isolate the dimeric half-filled molecules† from any unreacted globin chains and heme-containing subunits. Unreacted dimeric β-globin chains were removed after precipitation by dialysis against a 0.1 M potassium phosphate, pH 7.4.

Determination of Sulfhydryl Reactivity. The Ellman method (6) was used for the determination of sulfhydryl reactivity of β-globin as described before (1); for the heme-containing protein, a method modified from that described by Jacob *et al.* (7), using 5,5'-dithiobis(2-nitrobenzoic acid) was used. A volume of 3.0 ml of the reconstituted met β-subunit at a concentration of approximately 20 μM was pipetted into sample and reference cuvettes with 10 mm light paths. One-tenth milliliter of dithionitrobenzoate solution (39.6 mg/100 ml of 0.1 M potassium phosphate, pH 7.0) was added to the sample solution and mixed, while 0.1 ml of buffer was added to the reference solution. Absorbance at 412 nm was recorded on a Cary 14 spectrophotometer when the maximum was reached, usually within 10 min. For titration of total sulfhydryl groups, 0.1 ml of a 30% sodium dodecyl sulfate solution was added to the above sample and reference solutions. The maximum absorbance was obtained as soon as mixing was complete. Alternatively, sodium dodecyl sulfate solution was added before the dithionitrobenzoate; both procedures gave identical results.

Reduction of Methemoglobin. As a control experiment for the reduction procedure, oxyhemoglobin was converted to methemoglobin by reacting with 1.5 mol of potassium ferricyanide per mol of heme for 15 min. The oxidizing reagent was then removed by passing the reaction mixture through a Bio-Gel P-2 column equilibrated with 0.1 M potassium phosphate, pH 7.4. Methemoglobin was reduced by the nonenzymatic method of Kajita *et al.* (8), by using NADH and phenazine methosulfate. The general procedure adopted for our experiments was as follows: a 20-fold molar excess of NADH and a catalytic amount of phenazine methosulfate, 2.5×10^{-2} times the molar quantity of methemoglobin on heme basis, were added to the methemoglobin solution. The solution was mixed by a gentle swirling motion for 5 min while under a stream of oxygen. The reducing agents were then removed by filtration through a Bio-Gel P-2 column equilibrated with the appropriate buffer.

Oxygen Equilibrium Measurement. The experimental and computing procedures of Benesch *et al.* (9) were used to obtain the oxygen equilibrium curves. The equilibrium measurements were carried out at 20° and with hemoglobin concentration in the range of 50–500 μM (on a heme basis). A tonometer with a cuvette of 10 mm light path was used. Deoxygenation of oxyhemoglobin solutions was accomplished by a mechanical vacuum pump. Absorption spectra from 500 to 600 nm were recorded on a Cary 14 spectrophotometer. The buffers used were 50 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane (Bis-Tris) for pH 6.6–7.5, and 50 mM Tris-HCl for pH 8.0–8.5. The pentacyclohexylammonium salt of 2,3-diphosphoglycerate was converted to the free acid form by treatment with Dowex 50 resin according to Benesch *et al.* (10), and adjusted to pH 7.0 by the addition of a 1.0 M Tris solution before use.

RESULTS

Subunits reconstituted from the separated globin chains were recombined to form hemoglobin either by combination of stoichiometric amounts of reconstituted subunits to yield $(\alpha_R\beta_R)_2$ or by combination of each of the reconstituted subunits with a stoichiometric amount of the complementary native one to give $(\alpha_N\beta_R)_2$ and $(\alpha_R\beta_N)_2$. Functional properties of each of these reconstituted hemoglobins were studied with regard to cooperativity in oxygen binding, oxygen affinity in the presence and absence of P_2-glycerate, and the Bohr effect.

Reduction by NADH/Phenazine Methosulfate. Since reconstitution of the subunits was brought about by the addition of hemin to the globin chains, reduction of the ferric hemeprotein was necessary to functional studies. The separated ferri-subunits, native as well as reconstituted, were not reduced by NADH/phenazine methosulfate. Reassembled ferric $(\alpha_R\beta_R)_2$ or mixed state ferri-ferrous hemoglobins $(\alpha_N\beta_R)_2$ and $(\alpha_R\beta_N)_2$ are reduced to an extent varying between 85 and 95%, and similar experiments using native ferri-hemoglobin also show an average of 95% reduction.

Oxygen Equilibrium. Oxygen equilibrium curves for $(\alpha_R\beta_R)_2$, $(\alpha_N\beta_R)_2$, and $(\alpha_R\beta_N)_2$, as shown in Fig. 2, are sigmoidal, showing cooperativity in the binding of oxygen. The oxygen binding parameters for these reconstituted hemoglobins are

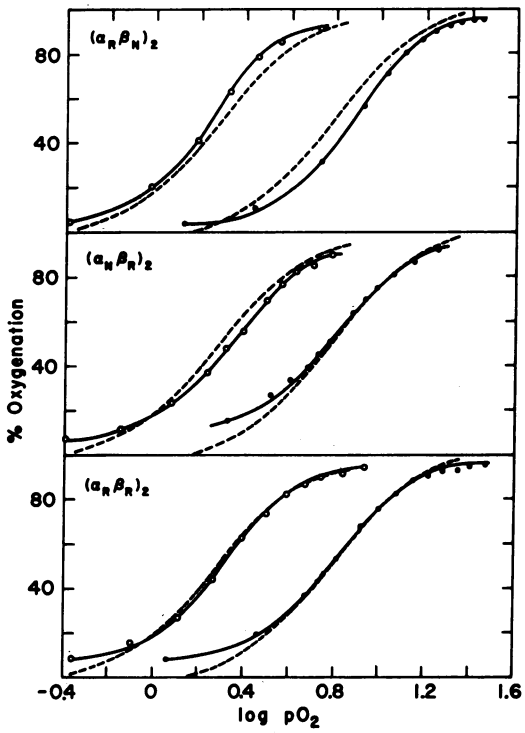

FIG. 2. Oxygen binding curves of reconstituted hemoglobins in the presence and absence of P_2-glycerate. The oxygen pressure pO_2 is expressed in mm of mercury (1 mm of mercury = 133 Pa). Hemoglobin concentration, 50 μM; buffer, 50 mM Bis-Tris, pH 7.4; temperature, 20°. Broken lines, control experiments with Hb A; O, in the absence of P_2-glycerate; ●, in the presence of 0.5 mM P_2-glycerate.

summarized in Table 1. The value for the Hill coefficient, n, is 2.55 for $(\alpha_R\beta_N)_2$, 2.50 for $(\alpha_N\beta_R)_2$, and 2.35 for $(\alpha_R\beta_R)_2$. These values are somewhat lower than those we obtain with native hemoglobin that has never been oxidized (2.8–3.0), but control experiments with native ferri-hemoglobin that had been reduced as above gave an average value of 2.65.

The oxygen affinity of these reconstituted hemoglobins, as measured by the value of log P_{50} (Tables 1 and 2), is virtually identical to that of the native hemoglobin. Furthermore, the values are in good agreement with those observed by Benesch *et al.* (10) and Huestis and Raftery (11) using native hemoglobin under comparable experimental conditions.

Effect of P_2-Glycerate on Oxygen Affinity. It is apparent from Fig. 2 that in the presence of P_2-glycerate all reconstituted hemoglobins show a decrease in oxygen affinity, and to the same extent as that shown by the native hemoglobin. Table 2 compares the oxygen affinity of these hemoglobins in the presence and absence of 1 mM P_2-glycerate in terms of the log P_{50} values.

Table 1. Oxygen equilibrium parameters of reconstituted hemoglobins

Hemoglobin	Hill coefficient	Log P_{50}
Hb A	2.65 ± 0.27	1.10
$(\alpha_R\beta_N)_2$	2.55 ± 0.25	1.09
$(\alpha_N\beta_R)_2$	2.50 ± 0.16	1.05
$(\alpha_R\beta_R)_2$	2.35 ± 0.06	1.08

Hemoglobin samples, in concentration ranges between 5 and 50 μM, were in 50 mM pH 7.0 Bis-Tris buffer containing 1 mM P_2-glycerate. Oxygen binding experiments were carried out at 20°. P_{50} is the oxygen pressure (expressed in mm of mercury) at which 50% of the hemoglobin molecules are oxygenated. The Hill coefficients are given ± standard deviation.

Table 2. Effect of 2,3-diphosphoglycerate on oxygen affinity of reconstituted hemoglobins

Hemoglobin	Log P_{50}	
	Buffer	Buffer + 1 mM P_2-glycerate
Hb A	0.30	0.80
$(\alpha_R\beta_N)_2$	0.34	0.86
$(\alpha_N\beta_R)_2$	0.36	0.77
$(\alpha_R\beta_R)_2$	0.31	0.79

Concentration of hemoglobin samples was approximately 50 μM. The buffer was 50 mM Bis-Tris, pH 7.4. Oxygen binding experiments were carried out at 20°.

The Bohr Effect. The Bohr effect, demonstrated as a plot of the change in oxygen affinity with change in pH, of the reconstituted hemoglobins is shown in Fig. 3. Results obtained with native hemoglobin are included for comparison. It can be seen that the reconstituted hemoglobins show the same pattern of changes in oxygen affinity as the native hemoglobins over the pH range studied, 6.0–8.5.

Other Properties. The absorption and circular dichroism spectral curves over the entire accessible spectrum, sulfhydryl reactivity, sedimentation coefficients, and behavior on gels of the reconstituted hemoglobins are all experimentally indistinguishable from the corresponding properties of native hemoglobin.

DISCUSSION

Based on their oxygen binding properties, and on the response of the allosteric effector P_2-glycerate, the human hemoglobins reconstituted by the two distinct routes described above are native. Moreover, the minor discrepancies in physico-chemical properties of the reconstituted β-subunit encountered in previous methods of preparing β-globin (which, however, generated significant departures from native *functional* properties) have been entirely resolved. Every criterion thus far applied reveals functional and structural identity between the native and reconstituted proteins. Given the numerous examples of departures from normal values for the log P_{50} values in the absence and presence of organic phosphates, for the Bohr effect, and for the Hill coefficient, all occasioned by single amino acid substitutions [see, for example, the recent review of Baldwin

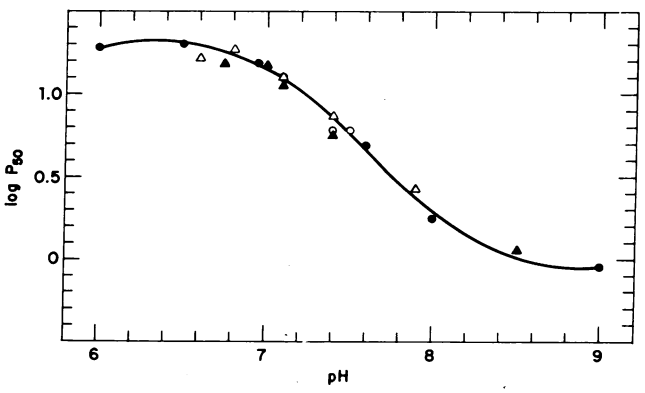

FIG. 3. Bohr effect of reconstituted hemoglobins. Hemoglobin concentration, 50 μM; P_2-glycerate, 0.5 mM; buffer, at pH 6.0–7.5, 50 mM Bis-Tris; at pH 7.5–9.0, 50 mM Tris·HCl; temperature 20°. ●, HbA; O, $(\alpha_R\beta_R)_2$; ▲, $(\alpha_R\beta_N)_2$; △, $(\alpha_N\beta_R)_2$.

(12)], it appears justified to assume that the fine structure of the native tetrameric molecule is closely or exactly approximated in the reconstituted hemoglobins.

A key step required to perform tests of biological function is the reduction of the met forms, which are obligatory products of reconstitution when hemin or cyanoheme is added to any separated globin or half-filled intermediate. The phenazine methosulfate/NADH redox couple (8) we have employed has the interesting and important property that it does not significantly reduce the separated α- and β-subunits or mixtures that fail to give correct combination of the ferri-subunits to ferrihemoglobin. This finding is in accord with earlier reports on the differences in reducing potential of the heme groups between the subunits in isolated states and in hemoglobin (13), and provides, also, a subtle check on the subunits.

With respect to the important question of yield, two key advances have been made that drastically increase the practicality of the reconstitution effort. One was the change to an acid pH of the buffer against which the freshly dissolved acid/acetone precipitate of β-globin is dialyzed and the maintenance of the protein at that pH prior to mixing with α subunit or hemin. The second was the realization that half-filled molecules could be prepared at quite high concentrations with no precipitation.

We have been satisfied for several years that fully native α-subunits could be reconstituted and, with recent preparations of β-globin, that the reconstituted β-subunits were also indistinguishable from native ones. However, the direct addition of ferriheme to the separated globin chains is accompanied, for still unexplained reasons, by massive precipitation except at very high dilutions. This has kept the yields at an unsatisfactory 5–10% for most preparations.

A reconstitution route through the half-filled molecules affords no such problems. The only present limitation on yield is that mixtures of aggregated α-globin and β^h give only about 50% combination to the purified dimer, $\alpha^\circ\beta^h$. In the case of the β-globin, combination with α^h gives a yield of better than 70%. In both cases, the final stoichiometric combination with ferriheme is uneventful.

In the pH interval 4–6.7, the separated globin chains and apohemoglobin undergo complex conformational transitions. It is possible that controlled combination of the globin chains in this pH interval can provide high yields of reconstituted hemoglobin without the use of untreated subunits. Above pH 6.7, the separated globin chains do not combine in the cold.

For the present, we would venture to suggest that experiments intended to test either globin chain should use the half-filled reconstitution route in preparation for structural or functional comparisons with native hemoglobin, whenever yield or ability to work at reasonably high concentrations are significant considerations.

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1. Yip, Y. K., Waks, M. & Beychok, S. (1972) *J. Biol. Chem.* **247**, 7237–7244.
2. Waks, M., Yip, Y. K. & Beychok, S. (1973) *J. Biol. Chem.* **248**, 6462–6470.
3. Bucci, E. & Fronticelli, C. (1965) *J. Biol. Chem.* **240**, PC 551–552.
4. Geraci, G., Parkhurst, L. J. & Gibson, Q. H. (1969) *J. Biol. Chem.* **244**, 4664–4667.
5. Gibson, Q. H. & Antonini, E. (1963) *J. Biol. Chem.* **238**, 1384–1388.
6. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77.
7. Jacob, H. S., Brain, M. C., Dacie, J. V., Carrell, R. W. & Lehmann, H. (1968) *Nature* **218**, 1214–1217.
8. Kajita, A., Noguchi, K. & Shukuya, R. (1970) *Biochem. Biophys. Res. Commun.* **39**, 1199–1204.
9. Benesch, R., Macduff, G. & Benesch, R. E. (1965) *Anal. Biochem.* **11**, 81–87.
10. Benesch, R. E., Benesch, R., Renthal, R. D. & Maeda, N. (1972) *Biochemistry* **11**, 3576–3582.
11. Huestis, W. H. & Raftery, M. A. (1972) *Biochemistry* **11**, 1648–1654.
12. Baldwin, J. M. (1975) *Prog. Biophys. Mol. Biol.* **29**, 225–320.
13. Banerjee, R. & Cassoly, R. (1969) *J. Mol. Biol.* **42**, 337–349.