EFFECTS OF ETHANOL AND 3,4,-DIHYDRO-2,2-DIMETHYL-2H-1-BENZOPYRAN-6-BUTYRIC ACID ON THE SOLUBILITY OF SICKLE HEMOGLOBIN

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SUMMARY: The effect of 3,4-dihydro-2,2-dimethyl-2H-1 benzopyran-6-butyric acid (DBA) on the solubility of deoxy-Hb S was evaluated by measuring saturation concentration, c_{sat}. Plots of c_{sat} versus DBA concentration in the presence or absence of ethanol gave two parallel lines, indicative of the additive fashion in which ethanol and DBA increase the solubility of deoxy-Hb S. At a DBA/Hb molar ratio of 10:1, c_{sat} was increased 16%. Ethanol alone increased c_{sat} comparably, but at a much higher molar excess (200:1). DBA had no effect on the oxygenation parameters of Hb S. Complementary solubility studies using the salting-out method showed that DBA had no effect on deoxy-Hb S, but decreased the solubility of deoxy-Hb A, oxy-Hb A and oxy-Hb S. Hence, no correlation exists between the effect of DBA on the solubility of deoxy-Hb S measured as c_{sat} and that measured by the salting-out technique.

The intracellular polymerization of deoxy-Hb S which occurs upon deoxygenation of SS erythrocytes is responsible for the sickling phenomenon. The intermolecular association of deoxy-Hb S molecules into microtubular fibers results from the profoundly diminished solubility of this species relative to oxy-Hb S (1,2). For in vitro studies of potential anti-sickling agents, either of two distinctly different solubility assays may be used. The salting-out technique utilizes the general property that any protein in high ionic strength buffer has its minimum solubility at the isoelectric point (3). The solubility of deoxy-Hb S may also be measured as the saturation concentration, c_{sat}, under conditions approaching physiological. The solubility determined by this technique reflects the concentration of monomeric Hb S in equilibrium

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Abbreviations: Hb, hemoglobin (A,S and F refer to adult, sickle and fetal Hb, respectively); SS, homozygous for Hb S; DBA, 3,4-dihydro-2-2-dimethyl-2H-1-benzopyran-6-butyric acid; MGC, minimum gelling concentration; Bis-Tris, N,N-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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with polymeric fibers prior to phase separation by centrifugation (4). Hence, c_{sat} is directly comparable to the decreased solubility within SS erythrocytes from which the pathology of sickle cell anemia ensues.

Attempts to increase the solubility of deoxy-Hb S by covalent modification have shown that a correlation exists between solubilities measured by salting out and those measured by MGC, which is proportional to c_{sat} (5), for pyridoxylated derivatives (6) but not for carbamylated derivatives (7,8). No systematic comparison has been made between the two types of solubility assay for such noncovalent inhibitors of deoxy-Hb S intermolecular aggregation as urea and guanidine hydrochloride (9,10), alkylureas (11), and amino acids (12,13). DBA, a synthetic analogue of the natural product xanthoxylol, has recently been reported to possess antisickling activity (14). Likewise, ethanol has been shown to decrease the extent of sickling of SS erythrocytes, as well as to increase the solubility of deoxy-Hb S in high ionic strength phosphate buffer (10).

The purpose of the present study was two-fold: (1) to evaluate the effects of DBA and ethanol, separately and in combination, on the physiologically relevant solubility, c_{sat}, of deoxy-Hb S and (2) to assess whether a correlation exists between the effect of DBA on the solubility of deoxy-Hb S as measured by c_{sat} and by the salting-out technique. Additionally, the effect of DBA on the solubility of deoxy-Hb A, oxy-Hb A and oxy-Hb S in high ionic strength phosphate buffer was evaluated.

Materials and Methods

Venous blood, anticoagulated with EDTA, was obtained from individuals with sickle cell anemia; Hb F content was < 4%. Packed erythrocytes, washed three times with 0.9% NaCl, were lysed either by mixing with 0.1 volume of toluene in the presence of glass beads or by the addition of 10 volumes of 5 mM KPO_4 buffer plus 0.5 mM EDTA, pH 7.4. In either case, the stroma was removed by centrifugation at 38,000 x g for 30 min. Hb concentration was determined by conversion to cyanmet-Hb, using the value 11.0 cm^{-1} (per heme) for ϵ_{mM} at 540 nm (15). Hemolysates obtained by toluene lysis were used for solubility measurements by the c_{sat} method, while those obtained by hypotonic lysis were used for solubility measurements by the salting-out method, as well as for evaluation of oxygen equilibrium curves. For c_{sat} determinations, lysates were concentrated to 32-36 g/dl by vacuum ultrafiltration, followed by dialysis at 4° against 0.15 M Bis-Tris buffer (pH 6.75 at 30°). Thereafter, the procedure followed was essentially as described previously (4), with the exception that an aliquot of DBA, dissolved either in water or 20% ethanol, was included in the appropriate system (see legend to Figure 1) so as to encompass DBA/Hb molar ratios in the range from 0 to 10.

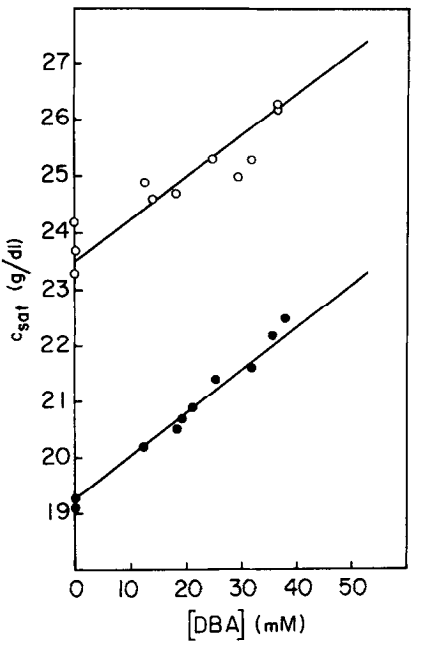
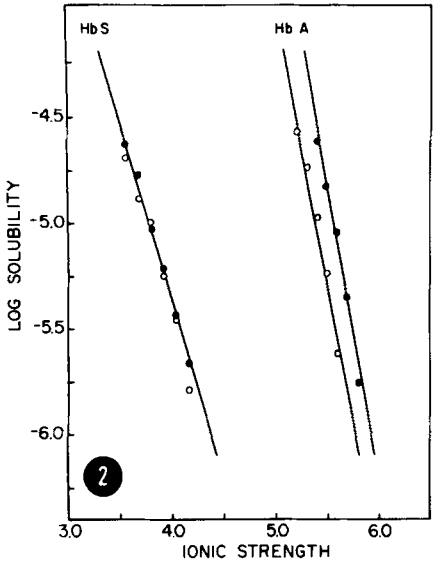


Figure 1. Solubility, c_{sat}, of deoxy-Hb S at pH 6.75 as a function of DBA concentration, in the absence (lower curve) or presence (upper curve) of 0.8 M ethanol. Initial concentrations, c_i, were in the range of either 23.5 to 24.3 g/dl (-ethanol) or 27.7 to 29.1 g/dl (+ethanol). Samples were equilibrated at 30° for 2 h, after which time polymeric (pellet) and monomeric (supernatant) phases were separated by centrifugation at 30° for 1 h at 300,000 x g. The data shown for each curve are the composite of three independent experiments; recovery was within 5% of c_i in each case.

Solubilities were determined as a function of ionic strength in phosphate buffer, pH 6.8, according to the modified procedure of Itano (16,17). Further details of the experimental design are given in the legends to Figures 2 and 3. Oxygen equilibrium curves were measured by the automatic recording method of Imai et al. (18)

Results

The effect of DBA on c_{sat}, in the absence or presence of ethanol, is shown in Figure 1. The two parallel lines obtained, one of which (+ethanol) is displaced upward from the other (-ethanol), indicate that either ethanol or DBA alone effectively increases the solubility of deoxy-Hb S. Comparison of the intercept for each line (corresponding to the control lacking DBA in either case) shows an increment in c_{sat} of 4.2 g/dl in the presence of 0.8 M ethanol. This solubility increase represents that due to ethanol alone. The parallel nature of the two lines as the DBA concentration was progressively increased in each



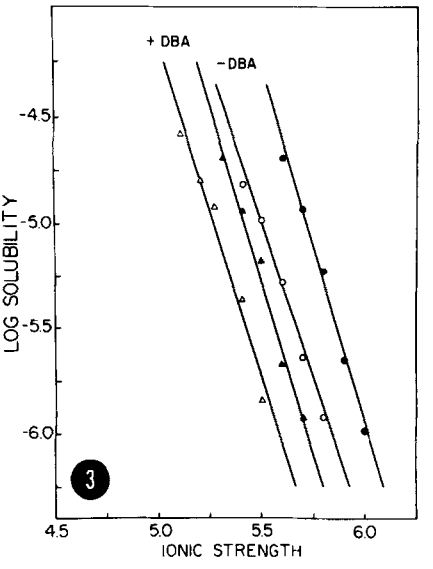


Figure 2. Solubility (moles/l) of deoxy-Hb A and deoxy-Hb S in the absence (●) or presence (o) of DBA as a function of ionic strength in phosphate buffer, pH 6.8. Where appropriate, the DBA/Hb molar ratio was 10:1. Reaction mixtures (4.0 ml), containing 8 mg of either Hb A or Hb S (as lysate) and 20 mg of sodium dithionite in suitably diluted stock phosphate buffer ($\mu = 8.0$), were incubated at 25° for 30 min and filtered with Millipore filters of 0.65 μm porosity. Hb concentration in the filtrate was determined spectrophotmetrically at 540 nm, using the value 10.28 cm^{-1} (per heme) for ϵ_{mM} of deoxy-Hb at this wavelength (15). For all filtrates the ratio A_{555}/A_{540} was in the range 1.22 to 1.26, verifying that the deoxy state was maintained (21) during manipulation of either species.

Oxygen equilibrium curves were measured at 20° with 12 μM solutions of Hb S in 0.1 M KPO_4 buffer plus 1 mM EDTA, pH 7.0. Oxygen saturation was evaluated by monitoring the absorbance change at 560 nm. A time interval of 45 min was required to effect the complete conversion of oxy-Hb S to deoxy-Hb S by exposure to humidified argon. In the presence of a 10-fold molar excess of DBA, the oxygenation parameters were virtually identical to the control(P_{50}= 10mm; n= 2.9). Other workers have reported a similar finding with ethanol (10). Hence, neither DBA nor ethanol appears to influence the solubility of Hb S by altering its oxygen affinity.

The solubilities of the deoxy conformation of Hb A and Hb S, in the presence or absence of DBA, as a function of ionic strength are shown in Figure 2. While the presence of DBA significantly decreased the solubility of deoxy-Hb A, it had no effect on the solubility of deoxy-Hb S. Similar salting-out curves for the oxy conformation of Hb A and Hb S are shown in Figure 3. In this case, the presence of DBA resulted in an appreciable decrease in solubility for both oxy-Hb A and oxy-Hb S.

Discussion

Although DBA and ethanol both increase, in additive fashion, the solubility of deoxy-Hb S measured as c_{sat} (Figure 1), the molar concentration of DBA required to effect the maximum increment in c_{sat} observed (16%) is much lower (0.04 M) than that required to produce a comparable increment with ethanol (0.8 M). Both agents presumably act by destabilizing noncovalent hydrophobic interactions in the polymeric fiber. However, the precise mechanism by which this occurs may be different for each. In the case of ethanol, the need for such a high molar concentration to effect increased solubility suggests that its mode of action may be nonspecific, i.e. mediated by an alteration of the dielectric properties of the solvent. By contrast, DBA is effective at a much lower molar concentration. Hence, it may act via a direct stereospecific interaction which disrupts an intermolecular contact site in the deoxy-Hb S polymer. In this regard, the side-to-side contact 1β6 Val to 2β85 Phe and 2β88 Leu observed in

the x-ray structure of crystals of deoxy-Hb S composed of double strands of molecules (19) is especially intriguing since DBA resembles phenylalanine structurally. However, until this contact site is shown unequivocally to be the same in the polymeric fiber, a steric interference with its formation by DBA remains merely speculative.

It is noteworthy that DBA had no effect on the solubility of deoxy-Hb S measured by salting-out, while it significantly decreased the solubility of deoxy-Hb A (Figure 2). This differential effect of DBA on the salting-out solubility of deoxy-Hb A versus deoxy-Hb S is remarkable inasmuch as these two Hb species are crystallographically isomorphous (19). This behavior may reflect the existence of a subtle conformational difference in solution between deoxy-Hb A and deoxy-Hb S. A similar distinction has previously been observed for oxy-Hb A versus oxy-Hb S with respect to the property of mechanical instability (20). It should also be noted that others have shown that the presence of ethanol increased the solubility of deoxy-Hb S measured by salting out (10).

In addition to decreasing the salting-out solubility of deoxy-Hb A (Figure 2), DBA also decreased the solubility of both oxy-Hb A and oxy-Hb S as measured by the salting-out technique (Figure 3). It would appear that for these three Hb species, DBA alters either or both of the physicochemical properties known to stabilize proteins in solution, namely water of hydration and electrostatic charge. The unique behavior of deoxy-Hb S in the presence of DBA may accrue from both the charge difference imposed by the substitution of valine for glutamic acid at position $\beta 6$ and the difference in quaternary structure between deoxy- and oxy-Hb S.

In conclusion, although DBA acts as a noncovalent inhibitor of the polymerization of deoxy-Hb S (Figure 1), this effect does not occur when the solubility of this species is measured at high ionic strength (Figure 2). Therefore, especially when perturbants of noncovalent interactions are involved, the effect of any potential anti-sickling agent on the solubility of deoxy-Hb S measured by salting out should be interpreted with caution. Unless complementary

solubility studies by the more physiologically relevant c_{sat} method corroborate results obtained by salting out, there is no assurance that the latter type of solubility assay reflects the intracellular polymerization of deoxy-Hb S which underlies the sickling phenomenon.

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